

## Cytotoxic Effect of *Ganoderma Lucidum* to Peripheral Blood Cells

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### Abstract

**Background :** The *Ganoderma lucidum*(GL) has been used to prevent and treat various human diseases for long times in the oriental area. Polysaccharides and triterpenes are known to bioactive compounds in the GL. Some case reports presented the developing pancytopenia in patient with ingestion of GL. And author experienced same case. The purpose of this study was to investigate an cytotoxic effect of polysaccharides and triterpenoids as extracts of GL on hematopoietic stem cell or peripheral blood cells.

**Materials and Methods :** Twenty one SD rats (male,  $150 \pm 10$ g, 6weeks) were divided into control, polysaccharide (PS), and triterpenoid (TT) groups. They were administered orally with Polysaccharide and triterpenoid being extracted from GL. The doses of GL extracts to weight of animal were 1, 0.2, and 0.04 mg/g. The number of white blood cell (WBC) was counted serially at the 24hrs before administration of PS and TT, and at the 3hrs, 6hrs, 24hrs, 48hrs and 72hrs after administration. At the 72hrs after ingestion of GL, femur bone was dissected for observing the cellularity in bone marrow of animals. Human leukemia U937 cells and hematopoietic stem cells, TF-1  $\alpha$  were incubated with various concentrations of PS and TT (1, 10 and 50  $\mu$ g/ml). And the cell viability was calculated after staining with trypan blue exclusion. The cell growth inhibition of PG and TT in leukemia cell lines was measured by direct cell counting.

**Result :** Suppressed percents of PS groups with high, middle and low dose (1, 0.2, and 0.04 mg/g) to control group were -29 %, -30 %, -27.3 %, respectively in 3 hours after oral administration of PS. And percents of TT groups with the same dose were -27.1 %, -39 %, -28.9 %. As time goes, the number of WBC was becoming recovered in all groups with the exception of TT groups with low and middle dose. Cell growth and cytotoxicity were less inhibited by PS and TT in TF-1  $\alpha$  cells than in U937 cells according to a dose-dependent manner; 80, 60, and 50 % of the cells survived after exposure to 1, 10, and 50 g/ml of PS, respectively, for 48hrs. The PS and TT-treated U937 cells decreased 2.0 fold in cell numbers and increased 2.5 to 5.0 fold in cytotoxicity. At last, slides of bone section obtained from animals of three groups showed the appearance of hypercellularity, similarly in all of the three bone marrows.

**Conclusion :** PS and TT extracted from GL may have cytotoxic effect on peripheral blood cell. But hematopoietic stem cell may be preserved from extracts of GL. Until now, there is not found accurately what is the mechanism of cytotoxic effect on peripheral blood cells by extracts of GL. In future, further studies will be needed to discover the mechanisms about cytotoxic effect of GL extracts on peripheral blood cell.

**Key words :** *Ganoderma lucidum*, WBC, cytotoxicity, TF-1  $\alpha$ , U937

### Introduction

The *Ganoderma lucidum* *Reishi* (GL) has been used to prevent and treat various human diseases for long time in the oriental area.<sup>1)</sup> The phytochemical constituents of GL include polysaccharides, proteins, nucleosides, fatty acids, sterols, cerebroside, and triterpenes.<sup>2)</sup> The potent bioactive compounds are polysaccharides and triterpenes among the constituents of GL. They have been identified from its fruit bodies, mycelia, and spores.<sup>3)</sup> Recently, pharmacological and

clinical studies demonstrated that *Ganoderma* polysaccharides and triterpenoids had anti-tumor and immunomodulatory activities. And some reports demonstrated that anti-tumor effect represented to be mechanisms such as immune-related or direct cytotoxic activities by GL.<sup>4,5)</sup> In fact, some reports presented that anti-tumor activities of GL affected human leukemic cell and tumor cell.<sup>6,7,8)</sup> On the other side, toxic effect and decreased number of cells were observed in peripheral blood mononuclear cells after being treated with GL extracts.<sup>9,10)</sup> And some case reports were published that reversible aplastic anemia was developed after ingestion of GL.<sup>11,12)</sup> Also, Author experienced that pancytopenia of peripheral blood was developed in patients with oral

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intaking of GL. So author had becoming to practice experimental study about cytotoxic effect of GL to peripheral blood. And the purpose of this study was to investigate an cytotoxic effect of polysaccharides and triterpenoids as extracts of GL on hematopoietic stem cell or peripheral blood cells.

## Material and Method

### Chemicals

*Ganoderma lucidum* was donated from the patient that had suffered from pancytopenia after ingestion of GL. The 400 g weight of GL was powdered, sunk in 2.4 L of 50% ethanol for 2 hours and extracted with boiling for 3 hours. Hot water extracts was centrifuged (3,000 rpm, 3 minutes) and the solutions was freeze-dried to yield an extract powder (11.7 g). An extract powder was dissolved in 100 ml of 80% ethanol and centrifuged (3,000 rpm, 5 minutes). To eliminated lipid-soluble ingredients, the resulting precipitate was 2 times washed with diethyl acetate and dried to yield the crude polysaccharide extracts. The supernatant solution of 80% ethanol was concentrated under vacuum and heat to reduce by half volume. The reduced supernatant of triterpenoids-enriched ethanol soluble fraction was extracted with chloroform 2 times. The collected solutions was dried under vacuum and heat to obtained total triterpenoids extracts. The powders of the crude polysaccharide extracts and total triterpenoids extracts were dissolved in 2% aqueous tragacanth solution according to the administration concentrations of animals.

### Animals

Twenty one SD (Sprague Dawley) Rats (male,  $150 \pm 10$  g, 6 weeks) were purchased from Bio evaluation center, Korean Research institute of Bioscience and Biotechnology. The animals were maintained in laboratory with  $20 \pm 2$  °C  $\pm$  1 °C,  $45 \pm 10$  % humidity, and a 12hrs light/dark cycle. Animals were divided into control (C), polysaccharide (PS), and triterpenoid (TT) groups. Animals of control group were taken pure water (H<sub>2</sub>O) by mouth. Animals of PS and TT groups were taken polysaccharide and triterpenoid by

mouth respectively. PS and TT groups were divided into three subgroups according to the dose of GL extracts. In the part of high dose, animals were given to GL extracts 1mg per weight 1g of animals. In the subgroups of medium and low dose, were 0.2mg and 0.04mg per weight 1g of animals respectively. And the each subgroups were composed of three animals respectively. But control group was not divided according to the dose and was composed of three animals.

### Methods of measuring the count of WBC in animals

The number of WBC was counted after making intake by mouth of GL extracts in the animals. Whole blood of animals was collected at the 24 hours before administration of GL extracts, and at the 3hrs, 6hrs, 24hrs, 48hrs, and 72hrs after administration of that respectively. When the animal was placed in a restraining tube, the vein being founded on the tail was punctured with a 24 gauge needle. And the whole blood was collected in a EDTA (ethylenediamine tetraacetic acid) bottle with about 0.5-1.0 ml volume. The count of WBC was measured by BECKMAN COULTER (LH755 analyzer). COULTER<sup>®</sup> LH SERIES DILUENT and COULTER LYSE S<sup>®</sup> were used to reagents. I presented groups of experimental animals and schedule of checking the count of peripheral blood in Fig. 1.

### Group :

#### A. Control



#### B. Polysaccharide (PS)

: High dose 1mg/g vs medium dose 0.2mg/g vs low dose 0.04mg/g



#### C. Triterpenoid (TT)

: High dose 1mg/g vs medium dose 0.2mg/g vs low dose 0.04mg/g



Oral administration of GL extracts



Before 24hr ↑ After 3hr ↑ 6hr ↑ 24hr ↑ 48hr ↑ 72hr ↑ BM ↑

Fig. 1. Schedule of measuring count of leukocyte in the animal

#### **Preparation of bone section for identifying cellularity of hematopoietic stem cells in bone marrow of animals**

After whole blood of animal was collected at the 72hrs after administration of GL extracts, the femur bones of the animal was dissected in the control, PG and TT group by size 10 mm × 5 mm × 5 mm, respectively and fixed immediately in 10% formalin for 12 hours at room temperature. Fixed specimens were thoroughly rinsed in saline. All specimens were decalcified at 25° C using 10% EDTA and Hydrochloride during 3-4 days. Samples were then rinsed thoroughly in distilled water, dehydrated in a graded series of ethanol, and embedded in 60-62° C paraffin. Section (4 μm) were cut and stained with hematoxyline and eosin. All slides were comparatively evaluated using an Nikon Eclipse 80i microscope at × 200 magnification.

#### **Cell culture and cytotoxicity assay and cell growth assay**

Human leukemia U937 cell and hematopoietic stem cell, TF-1 α were obtained from the American Type Culture Collection (Manassas, VA, USA). U937 and TF-1 α cells were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) containing 10 % fetal bovine serum (HyClone, Logan, UT, USA). The dishes containing cells were kept in a 37 ° C humidified incubator with a mixture of 95 % air and 5 % CO<sub>2</sub>. At 1 day prior to the experiment, cells were plated into 60-mm dishes. For trypan blue exclusion assay, cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4 % trypan blue solution, and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min and examined under a light microscope. At least 300 cells were counted for each survival determination. TF-1 α and U937 cells were seeded at a density of  $1.5 \times 10^6$  and incubated with various concentrations of PG and TT (1, 10 and 50 μg/ml). Total cell number in each group was counted. The cell viability was calculated after staining with trypan blue exclusion. The cell growth inhibition of PG and TT in leukemia cell lines was measured by direct cell counting. And cytotoxicity of cell was measured by cell viability.

## **Result**

#### **Effect of the types and doses of GL extracts on the number of WBC in rats**

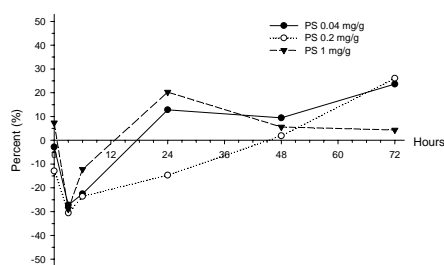
The mean percent was calculated from ratios of difference between counts of WBC (PS or TT group - control group) to counts of WBC in control group at the specific times. Animals of the three groups were administered polysaccharides dose-dependently (0.04, 0.2 and 1 mg/g). All the three groups revealed short-time WBC counts suppression and long-time recovery and hypercellularity. The short-time suppression was shown in 3 hours after oral administration. Suppressed percents to control group were -29 % for high dose, -30 % for middle dose and 27.3 % for low dose. As time goes, counts of WBC were becoming recovered and finally hypercellular after 72 hours for all groups. (Fig. 2A)

All animals of the three groups were administered triterpenoids dose-dependently (0.04, 0.2 and 1 mg/g). The short-time suppression was equally shown in 3 hours after oral administration. Suppressed percents were -27.1 % for high dose, -39 % for middle dose and -28.9 % for low dose. As time goes, counts of WBC were recovered but their degree were low and hypercellular response were not shown in low and middle dose groups until 72 hours. (Fig. 2B)

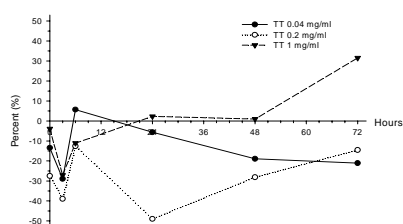
#### **Effect of PS and TT on cell growth and cytotoxicity in hematopoietic stem cells and leukemia cells**

The TF-1 α, hematopoietic stem cells as well as the U937, leukemia cell lines were used to study the effect of the polysaccharide and triterpenoid extracted from GL on cell growth and cell death. In Fig. 3A, the cells were treated with PS or TT of the time (24h and 48h) and the concentration (1 ~ 50 g/ml). PS and TT seems to be a slight growth and a little cytotoxicity in TF-1 α cells. U937 cells were treated with PS or TT of the time and dose dependent manner. PS inhibited cell growth and induced cytotoxicity in a dose-dependent manner; 80, 60, and 50 % of the cells survived after exposure to 1, 10, and 50 g/ml of PS, respectively, for 48h in U937 cells. The PS and

## A Polysaccharides



## B Triterpenoids



PS: polysaccharides, TT: triterpenoids

Fig. 2. Effect of the types and doses of GL extracts on the number of WBC in rats

A. According to low, middle and high dose (0.04, 0.2, 1 mg/g), the three groups of animals had oral administration of polysaccharides. Suppressed percents to control group were -29 %, -30 %, -27.3 % for high, middle and low dose, respectively in 3 hours after oral administration. As time goes, WBC counts were becoming recovered and finally hypercellular after 72 hours for all groups.

B. With regard to triterpenoids extracts, Suppressed percents were -27.1 %, -39 %, -28.9 % for high, middle, and low dose. As time goes, WBC counts were recovered but their degree were low and hypercellular response were not shown in low and middle doses groups until 72 hours.

TT-treated U937 cells decreased 2.0 fold in cell numbers and increased 2.5 to 5.0 fold in cytotoxicity. (Fig. 3B). Taken together, no significant differences were observed in TF-1  $\alpha$  cells, whereas PS or TT inhibited cell growth and increased cytotoxicity in U937 cells.

### Cellularity of bone marrow in the animal with oral administration of GL

The slides of animals showed that the counts of erythroblasts, myeloblasts, and megakaryocytes were increased independent of their groups. The bone marrow of femur were observed to be hypercellularity in animals, similarly. The images were showed in Fig. 4.

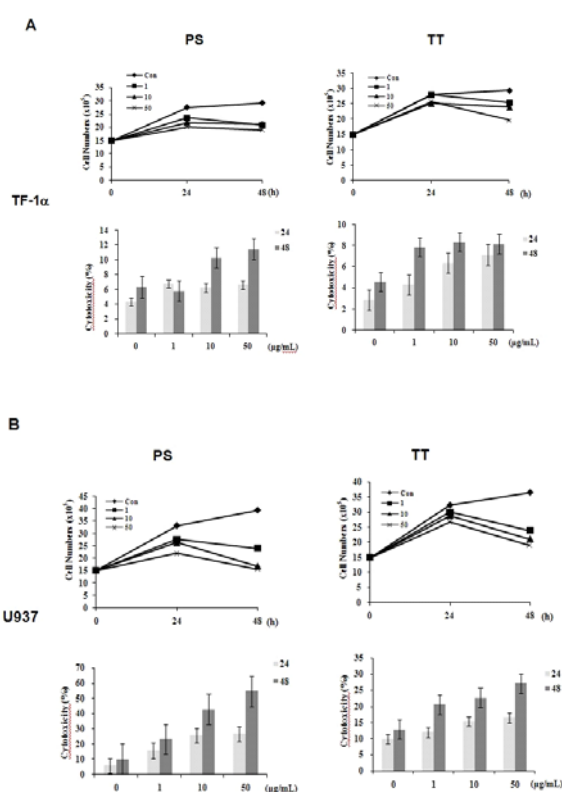


Fig. 3. Effect of PS and TT on cell growth and cytotoxicity in TF-1  $\alpha$  (A) and U937 (B). PS and TT treated TF-1  $\alpha$  cell and U937 cells in a concentration (1 to 50 g/ml) and time (24h and 48h) dependent manner. Untreated (control) or treated with the indicated condition of PS and TT and the cell number and the percentage of cell viability was measured by trypan blue assay as described in Materials and Methods. Cell cytotoxicity is presented as percentage of cell death of treated/untreated cells, for each condition.

### Discussion

This study revealed that more decreased count of WBC in PG and TT group than control group was expressed at the acute times (3hrs and 6hrs) after administration of GL extracts in animals, and the count of WBC was tended to be recovered at the 24hr after administration. This result suggests that the cause might be immunologic responses or intoxication of heavy metal or death of WBC by cytotoxic effects of GL. Thus the levels of cytokines including IL-2 and TNF- $\alpha$  were measured at the 24hrs before administration of GL extracts, and at the 3hrs, 24hrs, and 72hrs after. Data not shown. Because increasing level of IL-2 and TNF- $\alpha$  was proved to correlate with developing

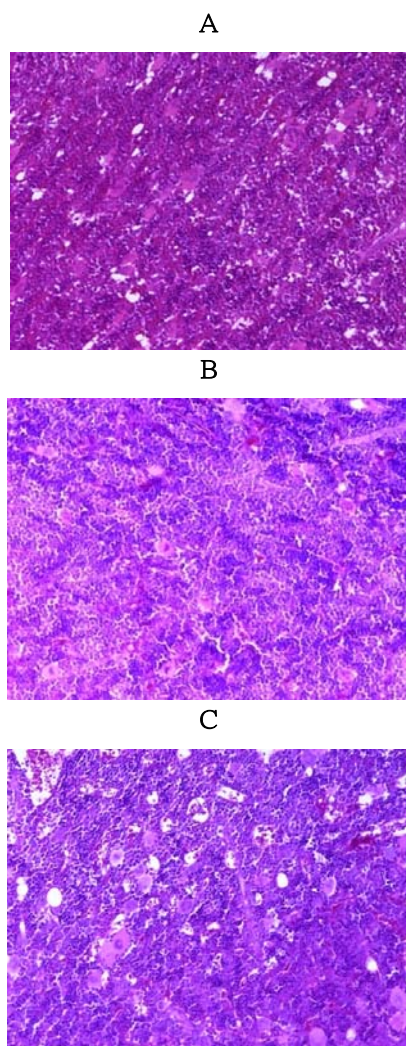


Fig. 4. Appearance of bone marrow at 96hrs after indigestion of GL in Animal.

A, B, and C expressed morphology of bone marrows of animals in control, polysaccharide, and triterpenoid group, respectively. The bone sections of animal show increased erythroblasts, myeloblasts, and megakaryocytes. they show the same appearance of hypercellular marrow (H&E, x200).

aplastic anemia.<sup>23,24</sup> But levels of cytokines including IL-2 and TNF- $\alpha$  were not increased. Taken together, thus suggest that cytotoxicity of GL might not correlated with immunologic reaction in limited to effect of decreasing count of WBC.

The level of heavy metal such as lead, cadmium, and Chromium which were known to have cytotoxic effects into peripheral blood was measured by Flameless method with Perkin Elmer AA800 model, Atomic absorption Spectrometer. Data not shown. All heavy metals were not detected in GL, which suggests that the decreasing count of

the leukocyte was not correlated with intoxication of heavy metals.

And next examination was whether or not the CD34 positive cell line (TF-1  $\alpha$ ) being affected by administration of GL extracts. TF-1  $\alpha$  cell line was not inhibited in growing of the cell in both PG and TT treated. But leukemic cell line (U937) was inhibited by GL extracts in contrast with TF-1  $\alpha$ . This study suggested that WBC of peripheral blood were died after attacked by GL extracts but WBC could be recovered because hematopoietic stem cells were stable in spite of attack of GL extracts and produced new blood cells in bone marrow of the animal. On the evidence, bone sections of animals show hypercellularity as reactivation of bone marrow in Fig. 4.

Recently, suppression of leukocyte had been documented by GL extracts. Gill et al<sup>9)</sup> reported that cell viability of leukocyte including T cell, B cell and peripheral blood mononuclear cells was associated with concentration of extracts from GL. The cell viability which indicate producing toxicity was decreased at higher concentration of extracts from GL. And these toxicity was associated with immune system including T and B cell. There were suggestions that suppression of WBC might be associated with immunologic response.<sup>9,10)</sup> But, this study is different from other studies in the point of having the evidence of decreased level in cytokines after administration of GL. Moreover polysaccharides and triterpenoids as two pharmacologic components of GL have function of anti-tumor and/or immunomodulatory effects.<sup>4)</sup> Until now, some mechanisms of immunomodulatory and anti-tumor effects have been documented. Some studies about immunomodulatory effect of GL reported that GL promotes the function of antigen-presenting cells, mononuclear phagocyte system, humoral immunity and cellular immunity.<sup>13,14,15,16)</sup>

On the other hand, GL has anti-tumor activity. Müller et al reported that GL extract has a activity against leukemia, lymphoma and multiple myeloma cells and may be a novel therapy for the treatment of hematologic malignancy.<sup>7)</sup> *Ganoderma* could inhibit the expression of uPA and uPAR, as well as the secretion of uPA. GL inhibit invasion of breast and prostate cancer cells by above

mechanism.<sup>17)</sup> And anti-tumor effects of GL extracts presented in sarcoma, lung cancer, hepatoma, and colorectal cancer etc too.<sup>8,18,19,20)</sup> And GL has antiangiogenesis as another mechanism of anti-tumor activity in sarcoma, adenocarcinoma, lung cancer and prostate cancer.<sup>21,22)</sup>

In this reports, I thought that death of leukocyte was associated with cytotoxicity by GL extracts. But I had limitation in our experimental study. First, GL extracts were not purified single compound. So other effects to animals and cell lines could be possible by another compounds which we did not know, in spite of the rare possibility. And concentration of single compound representing cytotoxic activities may be low level because single compound mixed with another compound in the total dose of GL extracts. Second, U937 cell line was used to control against TF-1  $\alpha$  as experimental cell line. But normal peripheral blood mononuclear cell was not tested by GL extracts as a control.

In conclusion, I suggest that the cause of decreased count of leukocyte may be cell death. But I do not know about accurate mechanisms of decreased leukocyte after administration of GL extracts. Only I suggest that mechanisms of decreased leukocyte may be associated with not immunologic responses but cell death.

In future, further research is required to determine mechanisms of cytotoxicities in peripheral blood and tumor cells after indigestion of GL extracts.

## 국문요약

### 영지버섯추출물의 말초혈액 세포에 미치는 세포독성 효과

영지버섯(*Ganoderma lucidum*)은 질병 예방 및 치료 목적으로 오랜 기간 사용되어 왔으며, 유효 성분은 크게 다당류와 트리테르펜류의 성분들로 알려져 있다. 본 연구는 자연산 영지버섯이 흰쥐에서 말초 백혈구수에 미치는 영향을 조사하고, 조혈모세포 줄기세포주에서 세포독성 여부를 조사하였다.

6주령 흰쥐 수컷 21 마리(150  $\pm$  10 g)를 대조군 3마리, 조다당류(PS) 처리군, 트리테르펜류(TT) 처리군, 각각

투여량 (1, 0.2, and 0.04 mg/g)에 따라 3마리씩 배정하였다. 백혈구수는 투여전과 후 시간에 따라 혈액을 채취하여 측정하였다. 투여 72시간 후에 쥐의 장골 골수 검사를 실시하였다. 인간 조혈모세포 줄기세포주인 TF-1  $\alpha$  와 인간 백혈병 세포주인 U937을 다양한 농도의 PS, TT을 처리하고 시간에 따라 세포독성에 대해 조사하였다.

백혈구수는 PS투여군의 경우 투여 3시간후에 대조군에 비해 용량에 따라 각각 -29, -30, -27.3% 으로, TT투여군에서는 -27.1, -39, -28.9% 으로 감소하였다. 세포배양 실험에서 U937의 경우 PS, TT 의 농도(1, 10, 50 g/ml)에 비례하여 세포수는 대조군에 비해 48시간후 각각 -39.1 ~ -60.4%, -34.4 ~ -47.5% 까지 감소하였으며, 세포독성은 각각 23.3 ~ 54.5%, 20.7 ~ 27.1% 까지 증가하였다. 반면 TF-1  $\alpha$  세포에 PS, TT를 처리하고 48시간후 세포수는 대조군에 비해 각각 -28.8 ~ -34.9 %, -13.6 ~ -33.3% 감소하고, 세포독성은 PS, TT의 모든 경우에 6.3 ~ 11.5% 로 변화가 미미하였다. 마지막으로 골수생검표본에서 대조군, PS, TT군 모두에서 세포과증식 양상을 관찰하였다.

결론적으로 영지버섯추출물인 PS와 TT는 백혈구수에 대한 억제작용을 가지고 있었으며, U937에 대한 세포독성은 확인되었으나, TF-1  $\alpha$  에는 경미하였다.

**중심단어:** 영지버섯, 백혈구수, 세포독성, TF-1  $\alpha$ , U937

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