Original

Effect of *Ganoderman lucidum* on Proliferation and Apoptosis of Pulmonary Microvascular Endothelial Cells *in vitro*

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Abstract : *Ganoderman lucidum* is a well-known Chinese crude and traditional herb that has been used in clinical settings in East Asia. One of its active components is a polysaccharide. The purpose of this study was to examine the effects of different doses of *Ganoderman lucidum* on proliferation and apoptosis in cultured rat pulmonary microvascular endothelial cells (RPMEC). The cell proliferation cycle was established for the cultured RPMEC and any changes in the cell cycle or apoptosis were observed using flow cytometry. Cell proliferation began four days after administration of *Ganoderman lucidum* (2.75 mg/ml) and peaked at the day 7. After 24h culture, the quantity of cells in G₀-G₁ cycle had decreased ($p<0.05$) while numbers in G₂-M cycle had increased ($p<0.05$) in the low dose group. The quantity of cells in G₀-G₁ cycle decreased ($p<0.05$) while numbers in S cycle increased ($p<0.05$) in the moderate dose group. The quantity of cells in G₀-G₁ cycle decreased ($p<0.01$), while numbers in G₂-M cycle ($p<0.01$) and S cycle ($p<0.05$) increased in the high dose group. No significant change in apoptosis was observed in any of the groups. Our results suggest that *Ganoderman lucidum* may stimulate the proliferation of cultured RPMEC, and therefore play an important role in healing injuries and accelerating repair.

Key words : pulmonary microvascular endothelial cells, *Ganoderman lucidum*, cell cycle, apoptosis

Introduction

Ganoderman lucidum, a well-known Chinese crude and traditional herb, has been widely used as a medicinal or health-promotion product in China and other East Asian countries¹⁻³⁾; its active components include a polysaccharide. *Ganoderman lucidum* is used for the treatment of hypertension, arteriosclerosis, hemorrhoids, symptoms of fatigue, cancer, diabetes, hepatitis, chronic bronchitis, arthritis, and asthma^{1, 4, 5)}. It is also used as a sedative and

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tranquilizer for dizziness and insomnia due to neurasthenia and as a tonic for symptoms of weakness or debility^{4,5)}. Previous approaches using *Ganoderman lucidum* showed an improvement in microcirculation and infusion volumes of vital organs in primary hypertension patients^{6,7)}. *Ganoderman lucidum* is also an effective inhibitory agent of platelet aggregation⁸⁾, however, its efficacy has not been confirmed and its exact mechanism of action has not yet been determined.

Microvascular endothelial cells play a very important role in hypertension, arteriosclerosis, thrombosis and other cardiac diseases. However, to our knowledge no reports evaluating the effect of *Ganoderman lucidum* on proliferation and apoptosis of microvascular endothelial cells *in vitro* have been published. To determine the effect on microvascular endothelial cells, we added a solution of *Ganoderman lucidum* at different concentrations into the culture medium and observed the effect on proliferation and apoptosis of microvascular endothelial cells using flow cytometry *in vitro*.

Material and Methods

Ganoderman lucidum

Ganoderman lucidum was extracted from tablets (Wakan Shoyaku Botany Institute, Tokyo, Japan) using hot water and freeze-drying. Each tablet contains 55 mg *Ganoderman lucidum* extracts (polysaccharide), equal to 1.375 g *Ganoderman lucidum* sporophores. The experimental solution of *Ganoderman lucidum* was produced by dissolving 50 *Ganoderman lucidum* tablets in 100 ml DMEM (Sigma Chemical Co., St Louis, MO), resulting in a solution containing a final concentration of 27.5 mg polysaccharide per milliliter, which was then agitated for 12 hours for complete dissolution at room temperature and filter sterilized using a 0.45 μ m filter. A control solution was prepared using the same matrix as the *Ganoderman lucidum* tablets (Wakan Shoyaku Botany Institute, Tokyo, Japan), and treated using the method described.

Culture of rat pulmonary microvascular endothelial cells (RPMEC)

We previously described the culture method for RPMEC⁹⁾. Briefly, male SD rats (weight range from 180~200 g) were chosen, injected intra-peritoneally with pentobarbital sodium (dose: 1 % 0.5 ml/100 g B.W.) and heparin (1000 μ /100 g B.W.). An *in situ* lung infusion with Hanks' balanced salt solution (HBSS) was performed. The side tissues of the rat's lung were cut into smaller pieces of 1 mm³, and 1-2 pieces per cm² were placed in a flask and cultured for 60-70 hours using DMEM supplemented with 20% bovine serum. After adequate cell proliferation, the tissue pieces were removed and the culture medium changed until the cells proliferated into a confluent monolayer.

Identification of RPMEC

The morphology of RPMEC was round, polygonal or spindle form (Giemsa staining), and stained positive for factor VIII-related antigen (DAKO) and CD31 (DAKO) using immunohistochemical staining. Non-endothelial cells represented less than 5 % of the total cells.

Examination by flow cytometry

The cell cycle and apoptosis of RPMEC were examined using the fluorescent stain PI

(propidium iodide). After 24 h cultivation, the cells were harvested by trypsinization, followed by addition of HBSS at pH 7.4 to adjust the cells to 10^4 /ml. For a total of 10^4 cells $5 \mu\text{l}$ of PI was used. A wavelength of 48 nm for red fluorescence was used for flow cytometry (Facs Calibur, Becton Dickinson, USA), and the percentage of cells for each cell cycle stage and apoptosis using a total of 5000 cells was analyzed using the MODFITLT for mac vl.01 and CELLQUEST programs.

Incubating RPMEC with Ganoderman lucidum

The quantity of RPMEC (2nd generation) was adjusted to 10^5 in 10 ml medium per culture flask. As previously reported⁶⁾, 0.1 ml *Ganoderman lucidum* solution (containing 2.75 mg polysaccharide) was added to each flask in the low-dose group, 0.4 ml (containing 11 mg polysaccharide) in the moderate-dose group, and 0.8 ml (containing 22 mg polysaccharide) in the high-dose group. Control solution (0.4 ml) was added to each culture flask in negative control group (the preliminary work showed that there were no differences between doses 0.1 ml, 0.4 ml and 0.8 ml of control solution). Culture medium was then added to each flask to a final volume of 10 ml and incubated for 24 hours before examining cell cycle and apoptosis by flow cytometry.

Determination of the cell growth curve

Day 1: A suspension containing 84 ml of cells (first generation) was prepared. Two milliliters of the suspension were added to each well of seven 6-well plates at a final concentration of 0.4×10^5 /ml. One of the 6-well plates contained three wells used as the control group (C-group) and the rest were the experimental group (E-group).

Day 2: Control solution (0.2 ml) was added to each well of the C-group and the same volume of *Ganoderman lucidum* solution was added to each well of the E group.

Cells were harvested from one six-well plate by trypsinization using 1 ml of 0.25% trypsin and then counted on days 3~8 and the final 6-well plate was counted on day 9.

The culture media was changed throughout the experiment and replaced as described for day 2. The experiment was repeated three times and the average number of cells in groups C and E were calculated for each time point. Growth curves for both the experimental and control groups were plotted.

Statistical analysis

The results of the experiments were statistically analyzed by rank-sum test and t test.

Results

Effect of Ganoderman lucidum on growth of RPMEC

Ganoderman lucidum accelerates the proliferation of RPMEC. Compared with the control group, the quantity of cells at days 4 and 8 had increased significantly ($p < 0.05$) while the quantity of cells on days 5, 6 and 7 increased to a more significant degree ($p < 0.01$, Fig. 1)

Effects of different doses of Ganoderman lucidum on the cell cycle of RPMEC in vitro

The results showed that compared with the control group, the quantity of cells in G_0 - G_1 cycle decreased ($p < 0.05$), while those in G_2 -M cycle increased ($p < 0.05$) significantly

Fig. 1. Effect of *Ganoderman lucidum* on growth of RPMEC. Compared with the control group, the quantities of cells at days 4 and 8 increased significantly ($p < 0.05$) and on days 5, 6 and 7 increased to a more significant degree ($p < 0.01$).

Table 1. Effect of *Ganoderma Lucidum* on the cell cycle of RPMEC *in vitro*

Group	Number	Cell Cycle Analyses with Flow Cytometry		
		G ₀ ~G ₁ (%) [‡]	G ₂ ~M (%) [‡]	S (%) [‡]
Low dose group	9	83.31* (80.24-84.88)	13.81* (11.43-14.60)	5.38 (3.69- 5.82)
Moderate dose group	9	79.93* (78.05-82.30)	13.03* (10.72-13.57)	7.30* (6.50- 8.87)
High dose group	9	76.61** (73.55-78.65)	14.01** (12.77-18.65)	8.61* (7.58-10.59)
Control group	10	86.86 (86.45-88.39)	9.92 (7.62-11.68)	4.61 (3.00- 6.61)

[‡]median (25%~75%)

Compare with control group * $p < 0.05$, ** $p < 0.01$

in the low-dose group. This suggested that mitosis of microvascular endothelial cells became active after the addition of *Ganoderman lucidum*. In the moderate-dose group, a significant reduction of the quantity of cells in G₀-G₁ cycle ($p < 0.05$) was observed. Cells in G₂-M cycle increased but not significantly when compared with the control group. Cells in the S cycle increased significantly ($p < 0.05$) revealing that a moderate-dose of *Ganoderman lucidum* promoted DNA synthesis of microvascular endothelial cells. Most significant was that the quantity of cells in G₀-G₁ cycle decreased ($p < 0.01$), while cells in the G₂-M cycle ($p < 0.01$) and S cycle increased ($p < 0.05$) in the high-dose group. This suggested that high doses of *Ganoderman lucidum* can promote the synthesis of DNA and mitosis of microvascular endothelial cells. The details are shown in Table 1.

Table 2. Effect of Ganoderma Lucidum on apoptosis of RPMEC *in vitro*

Group	Number	Apoptosis percentage (%) [*]
Low dose group	9	3.70 (2.66–5.22)
Moderate dose group	9	4.78 (2.42–6.16)
High dose group	9	4.96 (2.04–8.90)
Control group	10	2.64 (1.91–5.30)

^{*}median (25% ~ 75%)

Effect of Ganoderman lucidum on apoptosis of RPMEC in vitro

The results of flow cytometry showed that no significant difference of the percentage of cells undergoing apoptosis was found between groups receiving different doses and the control group. The details are shown in Table 2.

Discussion

The efficacy of *Ganoderman lucidum* as an anticancer and tonic agent has been recognized for many years. It has been reported to have the following pharmacological effects: anticancer, antihypertensive, immunomodulatory, neuroactive, antihistamine, stabilization of mast cells, suppression of cough, hypoglycemic action, reduction of smooth muscle contraction, and antihepatotoxic action against CCl₄^{5, 10-17}.

The efficacy of *Ganoderman lucidum* for cells *in vitro* has usually been studied in tumor cells. It has been demonstrated that *Ganoderman lucidum* constitutively inhibited transcription factors (e.g. nuclear factor kappa B and AP-1) in breast MDA-MB-231 and prostate PC-3 cancer cells, which resulted in the inhibition of expression of urokinase-type plasminogen activator (uPA) and its receptor uPAR. *Ganoderman lucidum* suppressed cell adhesion and cell migration of highly invasive breast and prostate cancer cells suggesting a role in the reduction of tumor invasiveness^{18, 19}). *Ganoderman lucidum* was also found to inhibit the growth of human hepatoma Huh-7 cells, but not Chang liver cells, a normal human liver cell line³). *Ganoderman lucidum* inhibited cell proliferation in a dose- and time-dependent manner, which might be mediated through up-regulation of p21/Waf1 and down-regulation of cyclin D1. Furthermore, *Ganoderman lucidum* could directly induce apoptosis in MCF-7 cells, possibly through an up-regulation of a pro-apoptotic Bax protein²). However, to our knowledge there has been no report to date on the pharmacological efficacy of *Ganoderman lucidum* in vascular endothelial cells.

Our study demonstrated that *Ganoderman lucidum* improves the proliferation of RPMEC. Cell proliferation began on day 4 after administration of *Ganoderman lucidum* (2.75 mg/ml), and peaked at day 7 when compared with the control. Flow cytometry results showed that *Ganoderman lucidum* stimulates the proliferation of RPMEC after 24 h of culture. Characteristically a low dose of *Ganoderman lucidum* (0.275 mg/ml) increased the quantity of cells in the G₂-M cycle significantly, a moderate dose (1.1 mg/ml) increased cells in the S cycle, and a high dose (2.2 mg/ml) increased cells in the G₂-M as well as S cycle. Thus it can be seen, *Ganoderman lucidum* can increase the synthesis of DNA and mitosis of RPMEC *in vitro*, and a high dose is the most significant in all experimental groups. However, no significant changes in apoptosis were found in any of the different dosage groups.

Our study suggested that the effect of *Ganoderman lucidum* on stimulating the proliferation of cultured RPMEC is useful in accelerating its repair after injury. It plays an important role in maintaining the smoothness and integrity of microvessel wall and preventing thrombosis.

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