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Polysaccharide of *Cordyceps sinensis* Enhances Cisplatin Cytotoxicity in Non–Small Cell Lung Cancer H157 Cell Line

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Abstract

Background and objective: The extracts of *Cordyceps sinensis* (Berk) Sacc (CS) have been used as a traditional medicine for centuries. However, few studies have examined the adjuvant action of CS in the treatment of non–small cell lung cancer (NSCLC). So the aim of this study is to investigate the adjuvant role of CS in the treatment of NSCLC. **Methodology:** The effects of the combination treatment of the polysaccharide-rich fraction of CS and cisplatin on H157 NSCLC cells were investigated through MTT assay for cell viability, lactate dehydrogenase and fluorescein diacetate and propidium iodide assay for cytotoxicity, and with flow cytometric analysis for apoptosis. The expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in H157 cells were detected by immunohistochemistry. **Results:** Compared with the cells treated with cisplatin alone, cell viability was significantly decreased and the expression levels of VEGF and bFGF protein were significantly reduced in the cells treated with a combination of CS and cisplatin. **Conclusion:** The current study indicates that the polysaccharide of CS inhibits tumor growth in NSCLC and that CS may be a potential adjuvant chemotherapeutic agent in NSCLC therapy.

Keywords

Cordyceps sinensis (CS), non–small cell lung cancer (NSCLC), adjuvant action, cytotoxic effects, Chinese traditional medicine

Introduction

Cancer is one of the major public health problems in the world. Lung cancer is the leading cause of cancer death, and non–small cell lung cancer (NSCLC) accounts for more than 80% of lung cancer.[1,2] NSCLC is resistant to radiation and standard chemotherapy agents, resulting in an overall 5-year survival rate less than 15%.[3] Hence, it is important to find adjuvant agents for the treatment of NSCLC to reduce chemotherapy resistance.

Cordyceps sinensis (Berk.) Sacc. (*C sinensis*, CS) has been used in traditional Chinese medicine to treat asthma, bronchial inflammation, and lung inflammation[4,5] for more than 300 years. It is also applied as an aphrodisiac, analgesic, immune modulator, free radical scavenger, and inhibitor of tumor growth.[6] Recently, the composition and biological activities of CS have been identified.[6-8] CS polysaccharide, the purified carbohydrate of CS, is one of its major active ingredients. CS polysaccharide can stimulate phagocytes, inhibit tumor development, and protect liver function.[9-11]

Other bioactive constituents from *Cordyceps* species include cordycepin, antibacterial and antitumor adenosine derivatives, ophiocordin, and L-tryptophan.[6,12] Recent studies indicated that the polysaccharides of CS could produce an antioxidant activity and that its nucleosides inhibit platelet aggregation and probably inhibit tumor growth.[13,14] Extracts from artificially cultivated fruit bodies of CS could scavenge reactive oxygen species by inhibiting malondialdehyde formation though peroxynitrite generator SIN-1.[4,15,16] The bioactive compounds involved in these activities including polysaccharides,

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modified nucleosides, and cyclosporin-like metabolites, which are produced by fungus and related species. The beneficial effects of CS on renal and hepatic function protection and immunomodulation-related antitumor activities are very promising and worth further investigation.[17,18] An increasing number of studies used cultured mycelia. However, more mechanism-based, disease-oriented pharmacological studies on CS are required to ensure its clinical efficacy for particular diseases.[18,19]

Currently, vascular endothelial growth factor (VEGF) has been of keen interest in NSCLC. VEGF, a potent stimulator of vascular permeability, leads to fibrin deposition that serves as a scaffold for new vascular network formation.[20] Furthermore, basic fibroblast growth factor (bFGF), a soluble heparin-binding polypeptide belonging to the fibroblast growth factor (FGF) family, is commonly found in malignant tumors. It is reported that bFGF has a mitogenic effect on endothelial cells, and it is a potent inducer of angiogenesis.[21,22] Therefore, VEGF and bFGF play an important role in the angiogenesis and metastasis of NSCLC.

Although many studies have demonstrated that the polysaccharide of *Cordyceps* has an antitumor activity in a variety of cancers, little is known about its role in NSCLC. The aim of this study is to investigate whether the polysaccharide of *Cordyceps* has an antitumor activity in NSCLC cells and its mechanism.

Materials and Methodology

Preparation of Polysaccharide From Cordyceps sinensis and Determination of Polysaccharide, Protein, and Mannitol Contents

Cordyceps sinensis was purchased from Jiangsu Province Hospital of Traditional Chinese Medicine, China. The CS is harvested from Xiaojin Town, China. Xiaojin Town is located in Sichuan Province at an altitude of 3800 to 4500 m. The identification of *C sinensis* was provided by Jiangsu Food and Drug Administration. Polysaccharide (LPS-rich fraction) was extracted from one of the anamorph strains of *C sinensis* by using the phenol–sulfuric acid method.[23] In brief, all samples were dried at 40°C for 24 hours before being ground into powder and then boiled in 10 volumes of distilled water at 95°C to 100°C for 2 hours. After vacuum filtration, the sediment was collected after 3 washes with 95% (v/v) ethanol (EtOH) and 1 wash with EtOH. The sediment was further dissolved in distilled water and the undissolved material was discarded. The soluble proportion was freeze-dried to obtain the polysaccharide powder.

The contents of polysaccharide, protein, and mannitol in the extracts were determined as reported previously.[23] For the contents of mannitol, 1 mL of the solution containing 0.2 mg of extracts was mixed with 1 mL of 0.015 mol/L sodium periodate for 15 minutes. After that, 2 mL of 5.5 mmol/L rhamnose and 4 mL of fresh Nash reagent (1000 mL of 2 mol/L ammonium acetate mixed with 2 mL of acetic acid and 2 mL of acetyl acetone) were added to the mixture and incubated in a water bath for 15 minutes. The absorbance of the mixture was measured by using an automated microplate reader (Bio-Rad, Tokyo, Japan) at 570 nm.

Cell Culture and Drug Treatment

NSCLC cell line H157 was purchased from the Shanghai Cell Bank of the Chinese Academy of Medical Science (admitted by American Type Culture Collection). The cells were maintained in RPMI 1640 (Gibco, Langley, OK) medium supplemented with 10% fetal bovine serum (Gibco) at 37°C in a humid atmosphere of air containing 5% CO_2. The supernatant was discarded 2 days after the cells attached to the culture flasks, and then the cells were maintained in serum-free RPMI 1640 medium. The cells were divided into 5 groups: CS alone (treatment with 0.5 μg/mL LPS-rich fraction of *C sinensis* alone); cisplatin alone (treatment with 0.05 μg/mL cisplatin alone); 0.1 CS + cisplatin (combination treatment with 0.1 μg/mL LPS-rich fraction of *C sinensis* with 0.05 μg/mL cisplatin); and 0.5 CS + cisplatin (combination treatment with 0.5 μg/mL LPS-rich fraction of *C sinensis* with 0.05 μg/mL cisplatin); control (Cont, same volume of phosphate buffered saline [PBS]).

Cell Proliferation Assay

Cells were seeded into 96-well plates at 5×10^3 cells/well and treated with the drugs indicated above for 24, 36, and 48 hours. The cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St Louis, MO). After treatment, the cells were washed twice with PBS and incubated with 25 μL MTT solution (5 mg/mL) per well for 4 hours. The formazan precipitate was dissolved in 100 μL dimethyl sulfoxide (Sigma-Aldrich), and the absorbance was measured in an ELISA reader at optical density (OD) 570 nm.

Cytotoxicity Assays

Lactate dehydrogenase (LDH) assay. The cytotoxicity of the drugs to cells was determined by using the LDH assay (Nanjing Jiancheng, China). The activity of LDH released into the medium from dead cells was an indication of the drug-induced cytotoxicity. The cell-free supernatants from treated H157 cells were collected and then measured by

using LDH assay kit in an ELISA reader at OD 440 nm according to the manufacturer's protocol.

Fluorescein diacetate (FDA) and propidium iodide (PI) staining. Cell viability of H157 cells was assessed by FDA and PI staining (Sigma). Green FDA staining showed live cells, and red PI staining showed dead cells. The cells were seeded to 96-well culture plates at a density of 2×10^5 cells/mL and then were treated with varied concentrations of drugs and incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 48 hours. Cells were rinsed with PBS thrice and incubated with 10 μL FDA (50 mg/L) and 2.5 μL PI (50 mg/L) at 37°C for 5 minutes. The cells were rinsed twice in PBS and examined under a fluorescence microscope (Nikon, Tokyo, Japan).

Flow Cytometry

Fluorescein isothiocyanate (FITC)–conjugated annexin V was used to detect the externalization of phosphatidylserine that occurred at an early stage of apoptosis. Cells were treated with different combinations of drugs for 24 hours and harvested for flow cytometry. Cells were incubated in a binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) containing a saturated concentration of FITC-annexin V and PI for 15 minutes at room temperature. After incubation, the cells were pelleted and analyzed in a fluorescence activated cell sorter analyzer (Becton-Dickinson, Franklin Lakes, NJ).

Immunostaining Analyses

Due to the key role of vascular endothelial cell factor in angiogenesis, we decided to explore the effect of polysaccharide from *C sinensis* on VEGF in NSCLC cells. The expression of bFGF in cells was examined by immunohistochemistry. Cells were fixed in 100% methanol and then incubated with a rat polyclonal antibody against human bFGF (BD Biosciences, San Jose, CA) at a dilution of 1:200 overnight at 4°C. Instead of primary antibody, PBS was used as a negative control. After washing, the cells were incubated with a horseradish peroxidase (HRP)–conjugated rabbit polyclonal antibody against rat immunoglobulins (Zymed, San Francisco, CA). Color development was carried out by using streptavidin-peroxidase complex (Strept AB Complex/HRP Duet Mouse/Rabbit; DAKO, Glostrup, Belgium) and 3,30-diaminobenzidine as a chromogen. The expression of VEGF was evaluated through immunofluorescence staining. Cells were fixed on a cover slip and incubated with a rat monoclonal antibody against human VEGF (BD Biosciences) at a dilution of 1:100 for 60 minutes at room temperature. After washing, cells were incubated with a cy3 fluorescent-labeled secondary antibody for 30 minutes and then examined under a fluorescence microscope (Nikon).

Gel Electrophoresis and Western Blot Analysis

RIPA buffer was used to extract the total protein from treated cells. For SDS-PAGE, 50 μg of protein was electrophoresed onto SDS polyacrylamide gel and then electrotransferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). After blocking, the membrane was probed with 1 of the following primary antibodies: rat polyclonal antibody against human bFGF, rat monoclonal antibody against human VEGF, and rat monoclonal antibody against β-actin (Santa Cruz, Santa Cruz, CA). HRP-conjugated antibody against rat IgG (Santa Cruz) was used as secondary antibody. Immunoreactivity signals were amplified using diaminobenzidine.

Statistical Analysis

The SPSS (11.0) statistic package was used for data analysis. Results were expressed as mean ± standard deviation. One-way ANOVA was used to test the statistical differences among groups with Dunnett's test for single or multiple comparisons between groups. P value less than .05 was considered as statistically significant.

Results

Content of Polysaccharides in CS

Protein and mannitol were from hot water extracts of *C sinensis* natural mycelium. The total yield of the complex of mycelium (g/100 g) was 36.21 ± 2.01. In this complex, the content (g/100 g) of protein, polysaccharides, and mannitol were 16.11 ± 0.51, 17.33 ± 0.81, and 33.11 ± 1.88, respectively. The contents of polysaccharides of extracts from cultural mycelia were significantly higher.

Cell Proliferation Analysis

The effects of polysaccharide from *C sinensis* on H157 cells' viability were examined through MTT assay as shown in Figure 1. Compared with Cont group, the OD of cells after treatment with drugs was significantly decreased (all $P < .05$); however, ODs of CS + cisplatin treated groups at 24, 36, and 48 hours were significantly lower than those of the cisplatin alone group (all $P < .05$). There was no significant difference between 0.5 CS + cisplatin group and 0.1 CS + cisplatin group. Compared with control group, the OD of CS alone group showed no statistically significant difference.

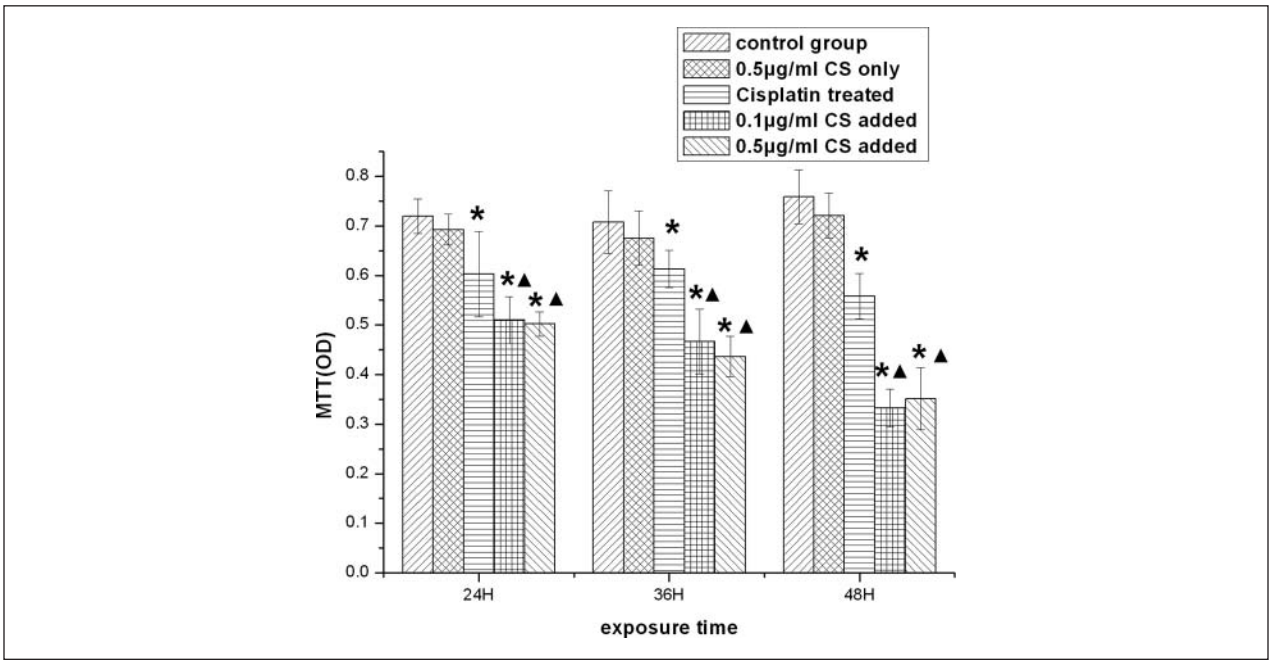

Figure 1. The effects of CS on H157 cells' viability at 24, 36, and 48 hours. Data are presented as mean ± standard deviation (n = 6). * indicate significant differences ($P < .05$) when compared with the control group; ▲ indicate significant differences ($P < .05$) when compared with the cisplatin alone group.

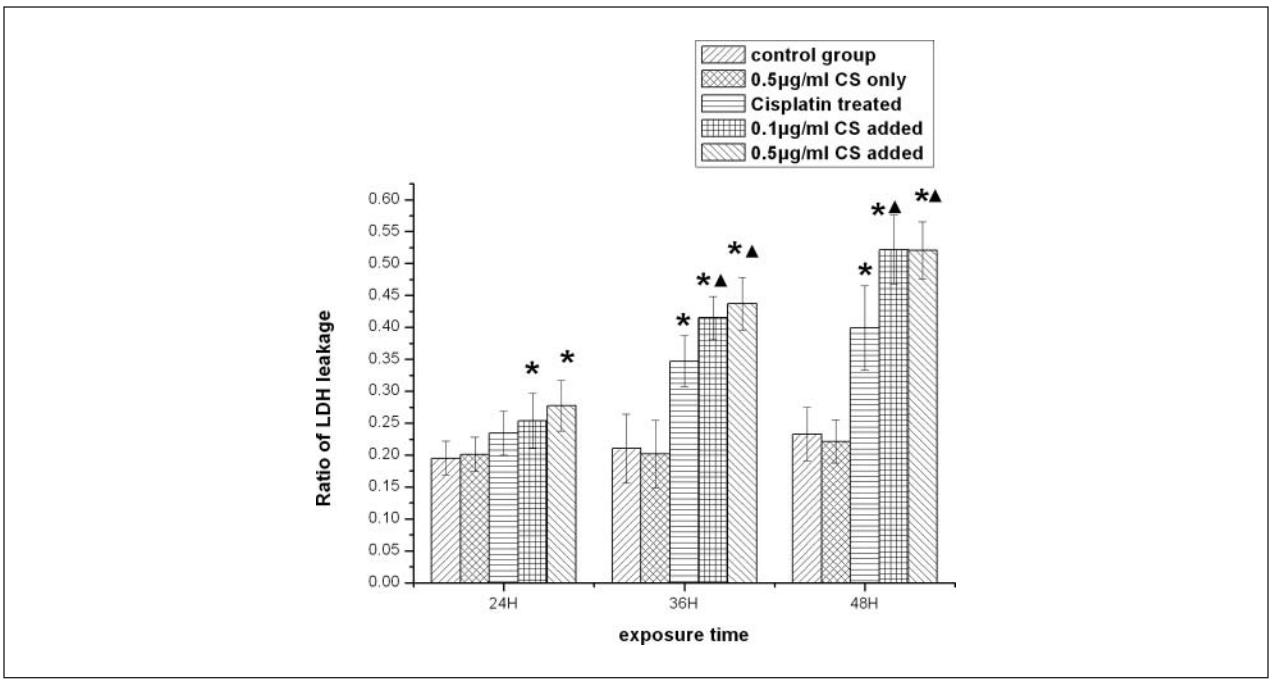

Figure 2. The effects of CS on LDH leakage in H157 cells at 24, 36, and 48 hours. Data are presented as mean ± standard deviation (n = 6). * indicate significant differences ($P < .05$) when compared with the control group;▲ indicate significant differences ($P < .05$) when compared with the cisplatin alone group.

Cytotoxicity Detection

The ratio of LDH leakage. Cytotoxicity was determined by LDH release from the treated cells as shown in Figure 2. Compared with the Cont group, the LDH released from CS + cisplatin group showed a significant increase in a time-dependent manner (all $P < .05$). Furthermore, the ratio of LDH leakage from the CS-alone group was

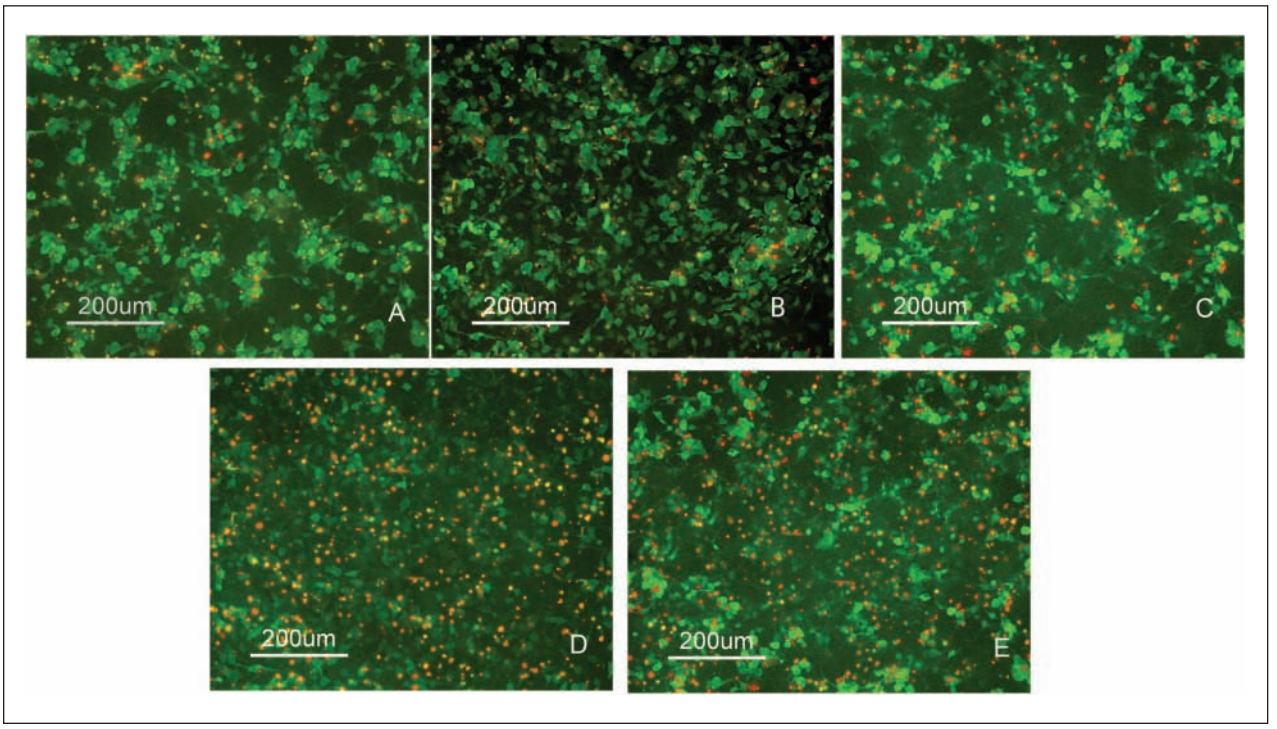

Figure 3. Fluorescent pictures of H157 cells with FDA and PI staining. Cells treated for 48 hours were stained with FDA (green) and PI (orange): (A) control group, (B) 0.5 μg/mL polysaccharide from *C sinensis*, (C) cisplatin-treated group, (D) 0.1 μg/mL CS added group, (E) 0.5 μg/mL CS added group. Scale bar = 200 μm

significantly increased at 36 and 48 hours compared with that of Cont or the cisplatin-alone groups.

Staining with FDA and PI. Plasma membrane damage was assessed by observing fluorescence photomicrographs of H157 cells stained with FDA and PI. Figure 3 shows representative fluorescence micrographs of each group of cells. The treatment of the polysaccharide from CS caused features of necrosis, such as cell shrinkage and loss of membrane integrity. The increase of red PI staining as well as the decrease of green FDA staining indicated that the number of dead cells had increased.

Apoptosis Detection

Figure 4 showed significantly increased apoptosis of cells treated with CS. After 24-hour incubation, a positive staining with annexin V was observed in cells treated with drugs. The combination treatment of CS with cisplatin induced more apoptotic cells than cisplatin alone ($P < .05$). The results of the 2 cell lines were similar.

Immunohistochemical Analyses

The expression levels of VEGF and bFGF protein in CS+ cisplatin groups were significantly decreased when compared with those of the cisplatin-alone group and Cont group (Figures 5 and 6).

Western Blot Analysis

Western blot analysis showed that the protein expression levels of VEGF and bFGF in CS + cisplatin groups were significantly reduced when compared with those of the cisplatin-alone group and Cont group (Figure 7).

Discussion

To date, only a few antineoplastic chemotherapeutic agents have shown reliable clinical activity in NSCLC, so NSCLC still remains one of the chemoresistant malignancies.[24] Among these drugs, cisplatin is the most frequently used in various combinations.[25]

Currently, new active antineoplastic drugs have been developed and become available for the treatment of NSCLC. Many studies have shown that the polysaccharide from CS possesses an antitumor activity. Possible mechanisms include enhancing immune function,[6] restraining differentiation,[26] and so on. Recent studies revealed that the polysaccharide from CS also had a potential effect of anti-angiogenesis, which might be one of the important mechanisms of its antitumor effects.[27,28] It is

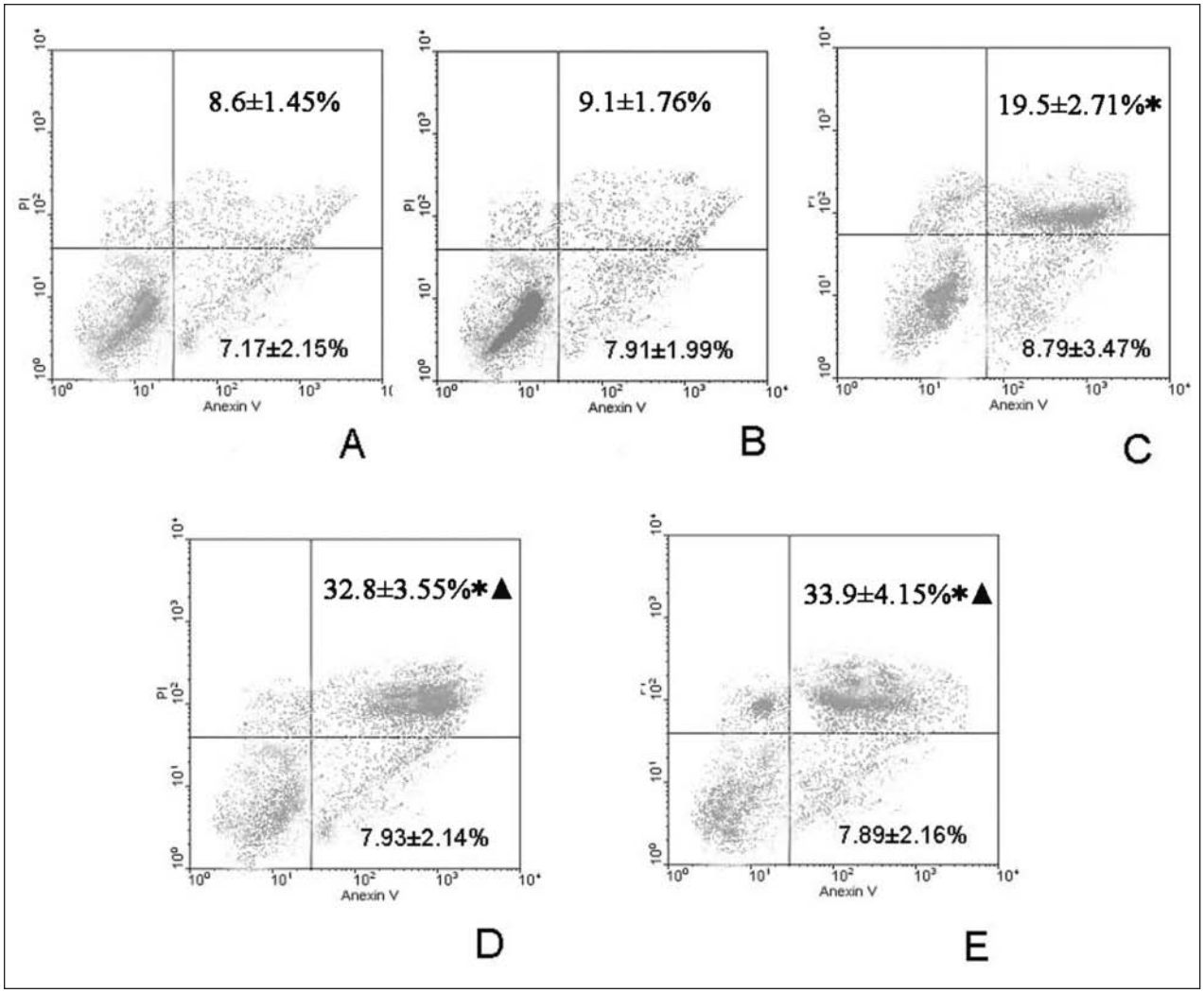

Figure 4. H157 cells apoptosis: (A) control group, (B) 0.5 μg/mL polysaccharide from *C sinensis*, (C) cisplatin-treated group, (D) 0.1 μg/mL CS added group, (E) 0.5 μg/mL CS added group

well known that angiogenesis depends on several aspects, such as the proliferation of endothelial cells for providing the necessary number of cells to grow vessels and the capability of the cells to migrate.[21]

Vascular endothelial growth factor, initially known as vascular permeability factor, is a 34 to 42 kD homodimeric protein that induces vasodilatation in a dose dependent manner in vivo. High VEGF expression and high angiogenic activity were noted in approximately 30% to 40% of the NSCLC cases. Basic fibroblast growth factor has pleiotropic effects in different cells and organ systems.[21] VEGF and bFGF are among the most important angiogenic factors. It was reported that assessments of circulating levels of VEGF and possibly bFGF were valuable tools for treatment planning and monitoring of treatment effect and relapse.[29] Due to the key role of vascular endothelial cell in angiogenesis, this study explored the effect of the polysaccharide from CS on NSCLC cells H157.

In this study, compared with the treatment of cisplatin alone, the combination treatment of CS with cisplatin decreased the cells' viability and induced higher numbers of dead cells. The protein and mRNA expressions of VEGF and bFGF in the cells receiving the combination treatment of CS and cisplatin were significantly lower than those in the cells that only received the treatment of cisplatin. These results demonstrated that the combination treatment of CS with cisplatin had better therapeutic effect than using cisplatin alone in NSCLC cells, suggesting that CS might play an adjuvant role in the cisplatin-induced cell death and anti-angiogenesis for NSCLC. These results also show that compared with a high dose of CS (0.5 μg/mL), a low dose of CS (0.1 μg/mL) could sufficiently provide an adjuvant effect for cisplatin.

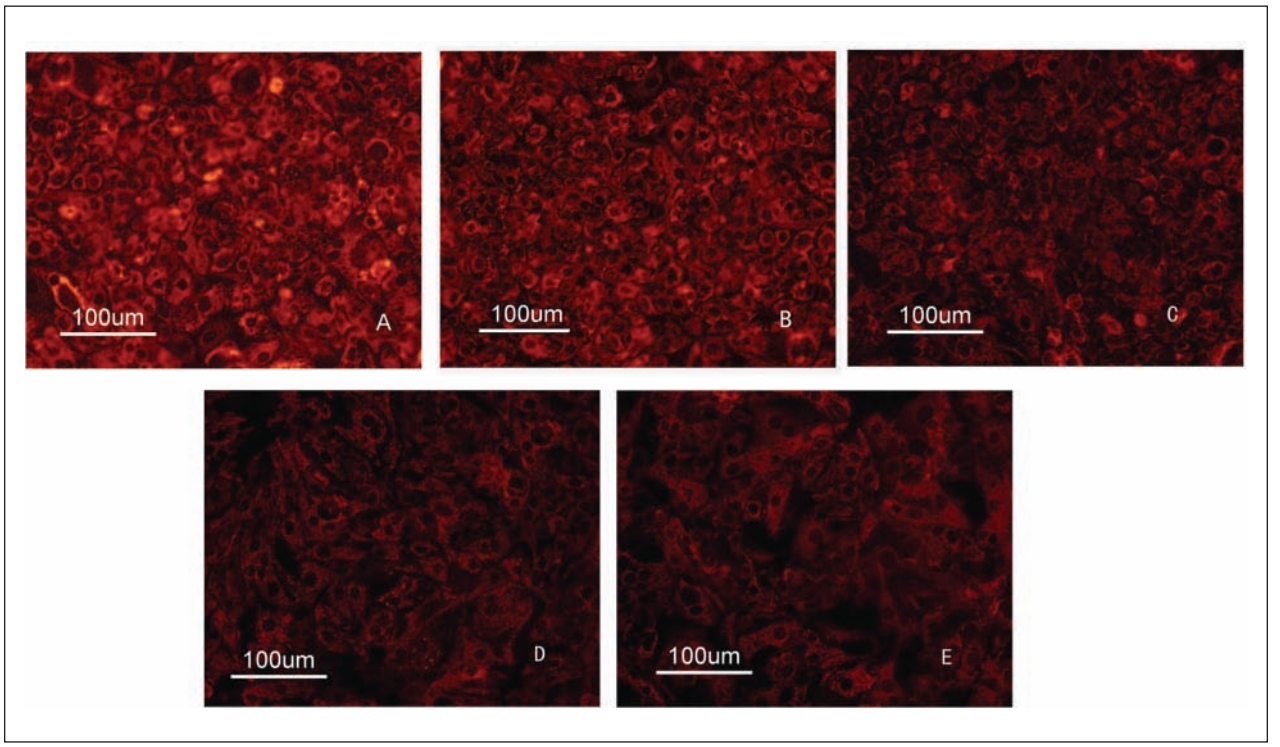

Figure 5. Expressions of VEGF in the H157 cells: (A) control group, (B) 0.5 μg/mL polysaccharide from *C sinensis*, (C) cisplatin-treated group, (D) 0.1 μg/mL CS added group, (E) 0.5 μg/mL CS added group. H157 cells in the control group (A) show higher expression of VEGF than the other groups. Notably lower expression of VEGF was found in (D), (E). Scale bar = 100 μm

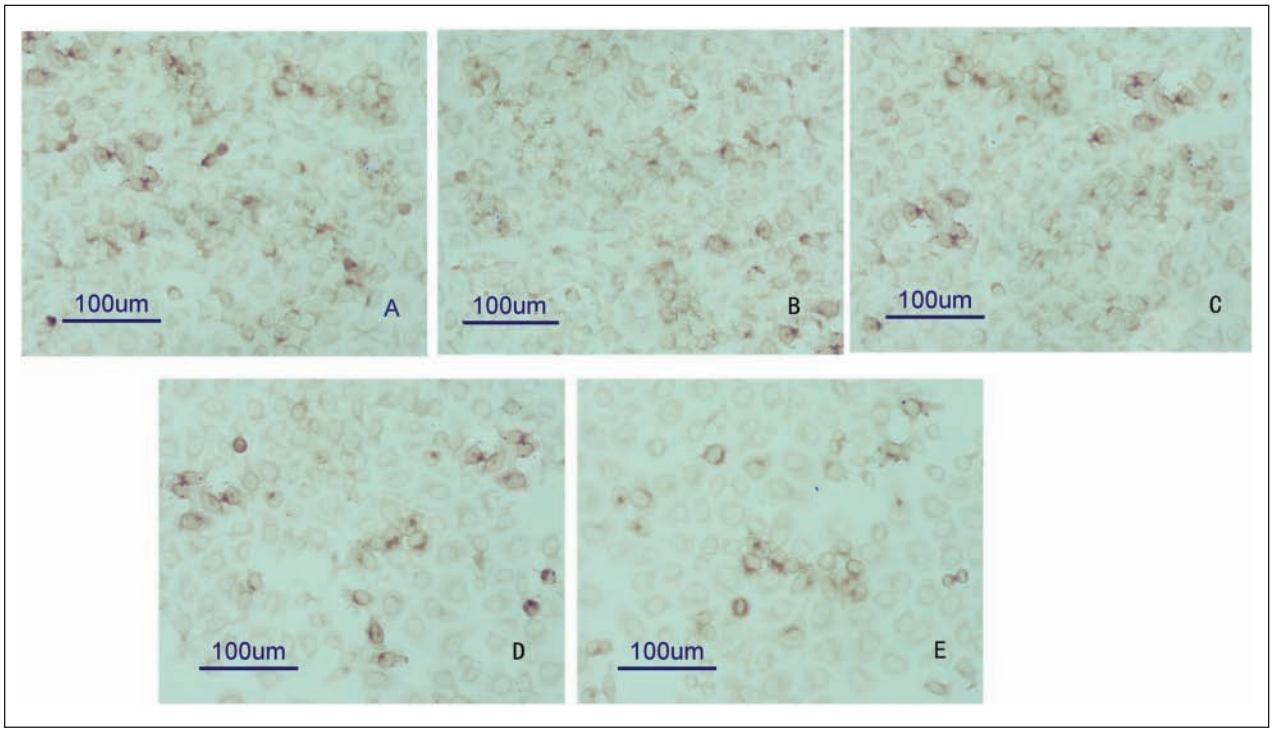

Figure 6. Expressions of bFGF in the H157 cells: (A) control group, (B) 0.5 μg/mL polysaccharide from *C sinensis*, (C) cisplatin-treated group, (D) 0.1 μg/mL CS added group, (E) 0.5 μg/mL CS added group. (A) showed higher expression of bFGF, and significantly lower expression of bFGF was found in (D) and (E). Scale bar = 100 μm

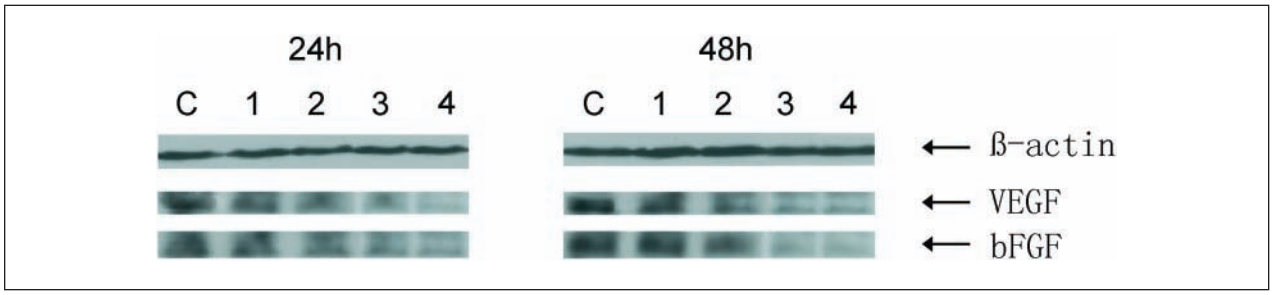

Figure 7. Western blot of VEGF and bFGF in H157 treated with CS for 24 hours
C, control group; 1, 0.5 μg/mL polysaccharide from *C sinensis* group; 2, cisplatin-treated group; 3, 0.1 μg/mL CS-added group; 4, 0.5 μg/mL CS-added group. C showed higher expression of both VEGF and bFGF, whereas their lower expressions were found in 3, 4.

Many studies have shown that VEGF and bFGF are 2 major factors influencing vascular permeability in tumor tissues.[20,22] VEGF and bFGF levels in tumor tissues and sera of NSCLC patients are negatively correlated with survival. In addition, 53% to 74% of NSCLC cells express VEGF and bFGF. Their expression levels are associated with higher T and N staging in the TNM classification, late stage of tumor, and poor survival. In the future, serum VEGF and bFGF levels may be used for personalized therapy.[21] Many studies have shown that the polysaccharide of CS could be absorbed well.[30-32] So we suggest that the polysaccharide from CS combined with cisplatin could provide additional benefit for inhibiting tumor growth in NSCLC.

Conclusions

The present study demonstrated that combination treatment of the polysaccharide from CS and cisplatin might enhance the therapeutic effect of cisplatin for NSCLC. Further investigations of the mechanisms involved are needed.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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