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ORIGINAL ARTICLE

Effect of *Lycium Barbarum* Polysaccharide Combined with Cisplatin on the Proliferation of Human Lung Cancer Cells

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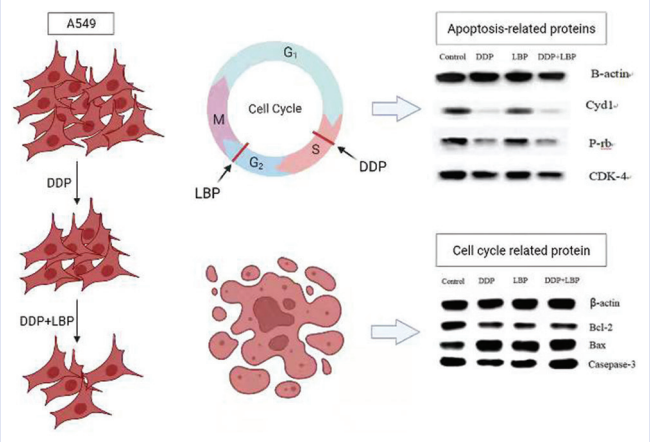
ABSTRACT

Background: *Lycium barbarum* polysaccharide (LBP) is a water-soluble polysaccharide extracted from *Lycium barbarum*. LBP exhibits potential pharmacological activity, including anti-cancer activities. However, so far, the effect of LBP in combination with cisplatin (DDP) on the proliferation of human alveolar adenocarcinoma cell line (A549) of non-small cell lung cancer (NSCLC) has not been reported. **Objectives:** In this study, we aimed to investigate the effect of LBP combined with DDP on the proliferation of A549 cells. **Materials and Methods**: The cells were divided into four groups: control group, DDP group, LBP group, and LBP and DDP combined group and Three parallel experiments were set up in each group. The survival rate of A549 cells and the effects of DDP and LBP alone or in combination was detected using the cell counting kit-8 (CCK-8) method. The activity of superoxide dismutase (SOD) was determined by xanthine oxidase method. The content of glutathione (GSH) was determined by colorimetry. The content of malondialdehyde (MDA) was determined by thiobarbituric acid method. The apoptosis, cell cycle phase, and the level of reaction oxygen species (ROS) formed were detected by flow cytometry. The expression of apoptosis-related proteins such as Bcl-2, Bax, and caspase-3 and cell cycle–related proteins namely, CDK4, cyclin D1, and p-Rb were detected via Western blot analysis. **Results:** DDP (≥ 6 mg/L) and LBP (≥ 8 mg/L) alone significantly inhibited the proliferation of A549 cells ($P < 0.01$), and LBP combined with DDP significantly enhanced the inhibitory effect on the proliferation of A549 cells ($P < 0.01$). DDP significantly reduced the activity of SOD and the level of GSH ($P < 0.01$), and significantly increased the level of MDA and ROS ($P < 0.01$). Compared with DDP group, the activity of SOD and the content of GSH and MDA in LBP and DDP combined group did not change significantly ($P > 0.05$), but the content of ROS decreased significantly ($P < 0.01$). DDP and LBP, alone and in combination, significantly promoted the cellular apoptosis ($P < 0.01$). They also significantly downregulated the expression of Bcl-2 and upregulated the expression of Bax and caspase-3, and the level of Bax/Bcl-2 was significantly increased ($P < 0.01$). DDP blocked the A549 cells in S phase, whereas LBP blocked the cells in G2/M phase. DDP and LBP combination significantly downregulated the expression of cell cycle regulatory proteins namely, CDK4, cyclin D1, and p-Rb ($P < 0.05$). **Conclusion:** LBP in combination with DDP inhibited the proliferation of A549 cells, and this combined effect is related to the expression of apoptosis-related proteins and the regulation of cell cycle mediated by cyclin D1-CDK4-Rb pathway.

Key words: Apoptosis, cell cycle, cisplatin, human lung cancer cells, *Lycium barbarum* polysaccharide

SUMMARY

- LBP can jointly promote DDP to inhibit the growth of human lung adenocarcinoma A549 cells, and LBP can enhance DDP-induced apoptosis of A549 cells.
- The combined effect is related to the regulation of cell cycle mediated by Cyclin-D1-CDK4-p-Rb pathway and the regulation of the expression of apoptosis-related proteins in cells, while the antioxidant capacity of LBP has no significant effect on the apoptosis-promoting effect of DDP.



Abbreviations used: LBP: *Lycium barbarum* polysaccharide; DDP: cisplatin; NSCLC: non-small cell lung cancer; SOD: superoxide dismutase; GSH: glutathione; MDA: malondialdehyde; ROS: reaction oxygen species. PVDF: polyvinylidene fluoride. SDS-PAGE: sodium dodecyl sulfate-polyacrylamide was used for gel electrophoresis; CDK: cyclin-dependent kinase

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INTRODUCTION

In China, lung cancer is a common malignant tumor with high rate of morbidity and mortality.[1] The incidence rate of lung cancer is increasing in China, mainly attributed to the deterioration of the environment.[2] In China, non-small cell lung cancer (NSCLC) has the highest rate of morbidity and mortality. At present, chemotherapy and surgery are the primary treatment options for lung cancer. *Lycium barbarum* polysaccharide (LBP) in combination with cisplatin (DDP) is one of the most commonly used and effective antineoplastic drugs in a clinical setting; however, it can cause side effects, such as nephrotoxicity,

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ototoxicity, and hepatotoxicity, which makes many patients give up treatment because their bodies cannot bear it.[3]

One of the most effective components extracted from *L. barbarum* is the LBP, which shows anti-aging effects, lowers blood cholesterol and sugar, enhances immune function, and shows antitumor effects.[4] Chen *et al*.[5] reported that LBP could inhibit the proliferation of gastric cancer cells by downregulating the expression of matrix metalloproteinases (MMPs). Previous studies have reported that LBP can inhibit and promote apoptosis of human prostate cancer cells (DU145) and mouse hepatoma cells (H22).[6,7] Furthermore, Miao *et al*.[8] showed that LBP significantly reduced the proliferation and growth of MGC-803 cells, and arrested the cells in G0/G1 and S phases.

According to a previous study,[9] LBP inhibited the DDP-induced apoptosis and autophagy in MlTc-1 cells. However, so far, there are no reports on the effect of LBP in combination with DDP on human lung cancer cells. Therefore in this study, we established a cell culture model of A549 cisplatin injury and studied the effects of LBP in combination with DDP on human lung adenocarcinoma cells (A549). The primary goal of this study was to evaluate the effect of LBP on the cell proliferation, oxidative damage, and apoptosis and cell cycle in A549 cells. The secondary goal of this study was to provide a theoretical basis for the action of LBP as an anti-cancer agent in the clinical setting and to enhance the sensitivity of DDP in chemotherapy.

MATERIALS AND METHODS

Materials and reagents

Human lung adenocarcinoma cells (A549) were purchased from the Cell Center of the School of Basic Medicine, Peking Union Medical College, Institute of Basic Medicine, Chinese Academy of Medical Sciences. The following chemicals were used in this study: freeze-dried powder for DDP injection (batch number: 406022CF; Qilu Pharmaceutical Co, Ltd); LBP (purity ≥99%) (Nanjing Zelang Pharmaceutical Technology Co, Ltd); Dulbecco's modified Eagle's medium (DMEM)/F12; CCK-8 kit; trypsin; apoptosis kit; cell cycle kit (Genview); malondialdehyde (MDA) kit; glutathione (GSH) and superoxide dismutase (SOD) (Nanjing Jiancheng Biological Engineering Research Institute); ROS detection kit; and cyclin D1, phospho-Rb, CDK4 (Biyuntian Institute of Biotechnology), Bax, Bcl-2, and caspase-3 antibodies (Bode Biotechnology Co, Ltd); fetal bovine serum (FBS, HyColone Inc); bicinchoninic acid (BCA) protein concentration determination kit; horseradish peroxidase-labeled anti-rabbit (mouse) immunoglobulin G (IgG); and β-actin antibody (Beijing Dingguo Changsheng Biotechnology Co., Ltd). All other reagents were of analytical grade. This research has been approved by the Ethics Committee(25-Mar-2021).

Instruments and equipment

In this study, the following instruments were used: FACSCalibur flow cytometry (American BD company); enzyme labeling instrument (American Bio-Tek company); electrophoresis instrument (American Bio-Rad company); WFZ UV-4802H ultraviolet visible spectrophotometer (Unocal Shanghai instrument Co, Ltd); and TE2000-M inverted microscope (Japanese Nikon company).

Methods

Cell culture

A549 cells were cultured in DMEM/F12 medium containing 10% FBS and double antibody (1%) in a humidified incubator (37°C and 5% CO_2). When the cells reached around 80%–90% confluency, the cells were harvested via trypsinization and subcultured according to the ratio of 1:3, and the cells in logarithmic growth phase were used for subsequent experiments.

Detection of cell proliferation by CCK-8 method

Effect of LBP and DDP alone on the proliferation of A549 cells

Briefly, A549 cells (100 μL, 1 × 10^5/mL) in logarithmic growth phase were inoculated into 96-well plates and incubated for 24 hours. After the cells adhered to the surface, the original culture medium was replaced with 100 μL of serum-free medium containing different concentrations of DDP (0, 1, 2, 4, 8, 8, 16, 32, 64, and 128 mg/L) or LBP (0, 2, 4, 8, 16, 32, 64, and 128 mg/L) with six multiple holes in each group. The cells were incubated for 24 hours in a humidified atmosphere (5% CO_2 at 37°C). After 24 hours, the culture medium was replaced with 100 μL medium containing 10% CCK-8 to each well and incubated further for 4 hours. The absorbance value (A) was read at 450 nm, and the cell survival rate was calculated using the following formula:

$$\text{Cell survival rate/\%} = \frac{A(\text{Experimental group})}{A(\text{Control group})}\ 100$$

Effect of LBP in combination with DDP on the proliferation of A549 cells

First, the inhibitory concentration (IC_{50}) of DDP and LBP was calculated according to the previous section, Effect of LBP and DDP alone on the proliferation of A549 cells. Based on the IC_{50} values, DDP and LBP were combined with two different concentrations (DDP: 6 and 12 mg/L; LBP: 8 and 16 mg/L). The cell survival rate was detected via CCK-8 method. In addition, the control group, DDP alone group, and LBP alone group were also set up. Briefly, A549 cells (100 μL, 1 × 10^5 cells/mL) in logarithmic growth phase were inoculated into 96-well plates. Each group consisted of six wells. After the cells were cultured in a humidified atmosphere (5% CO_2 at 37°C for 24 hours), the culture medium was replaced with 100 μL medium containing test compounds. The cell survival rate was calculated according to the formula given in the previous section.

Determination of intracellular protein concentration and antioxidant index of A549 cells

A549 cells in logarithmic growth phase (2 mL, 5 × 10^4 cells/mL) were inoculated into a 6-well plate. The cells were grown in a humidified incubator for 24 hours (5% CO_2). According to the optimal concentration in the section Effect of LBP in combination with DDP on the proliferation of A549 cells, the medium was replaced with medium containing LBP and DDP to continue the incubation for 24 hours. Subsequently, the cells were scraped and collected by adding 2 mL of cold phosphate-buffered saline (PBS) to the culture flask. The cell suspension was centrifuged at 1,000 rpm/min for 10 minutes, and the supernatant was discarded. The pellet was washed twice with 1 mL PBS and resuspend in 0.5 mL of PBS. The suspended cells were put into a cell crusher for crushing (30 Hz, 10 seconds). The cellular contents were centrifuged at 12,000 rpm/min for 10 minutes, and the supernatant was collected. The concentration of cellular protein, MDA, and GSH and SOD activity were determined according to the instructions provided in the kit.

Detection of ROS content in A549 cells by flow cytometry

DCFH-DA was diluted with serum-free DMEM/F-12 medium in the ratio of 1:1000 to make the final concentration of 10 μmol/L. According to the grouping seen in the section Effect of LBP in combination with DDP on the proliferation of A549 cells, A549 cells in logarithmic phase were inoculated into 6-well plates (2 mL,

5×10^4 cells/mL) and cultured in a humidified incubator at 37°C and 5% CO_2 for 24 hours. Subsequently, the cells were incubated with test compounds for 24 hours. Then the medium was discarded, and the cells were washed twice with PBS. Then 1 mL of diluted DCFH-DA was added to each well and incubated at 37°C for 20 minutes. The cells were washed thrice with serum-free cell culture medium to remove unbound DCFH-DA dye. The cells were harvested via trypsinization (1 mL of 0.25% trypsin without EDTA), centrifuged at 1,000 rpm/min for 5 minutes, after which the supernatant was removed, and the cells were re-suspended in 500 µL of PBS. The cells were screened by 200 mesh filters and collected in the flow cytometry tube. The excited light wavelength was 480 nm and the emission wavelength was 530 nm. The average fluorescence intensity of ROS was measured using CellQuest Pro software to express the relative content of ROS.

Detection of apoptosis rate of A549 cells by flow cytometry

A549 cells in logarithmic phase were inoculated into 6-well plates (2 mL, 5×10^4 cells/mL) and grown in a humidified incubator at 37°C and 5% CO_2 for 24 hours. According to the grouping in the section Effect of LBP in combination with DDP on the proliferation of A549 cells, the cells were incubated with test compounds for 24 hours, and the cells were digested with trypsin (1 mL of 0.25% trypsin without EDTA acted until the cells were loosely detached). The digestion was stopped with serum-containing medium and centrifuged at 1,000 rpm/min (4°C for 10 minutes) and the supernatant was discarded. The cells was resuspended in 1 mL of PBS and centrifuged at 1,000 rpm/min for 10 minutes. The supernatant was discarded and the process was repeated twice. The rate of apoptosis of A549 cells was determined by Annexin V-FITC double staining method.

Detection of cell cycle by flow cytometry

A549 cells in logarithmic phase were inoculated into 6-well plates (2 mL, 5×10^4 cells/mL) and grown in a humidified incubator at 37°C and 5% CO_2 for 24 hours. According to the grouping in the section Effect of LBP in combination with DDP on the proliferation of A549 cells, the cells were incubated with test compounds for 24 hours. The cells were then fixed with 70% ethanol for 24 hours (4°C) according to the instructions provided in the kit. The cells were analyzed by flow cytometry after staining with propidium iodide (PI).

Detection of protein expression in A549 cells by Western blot

A549 cells in logarithmic phase were inoculated into 6-well plates (2 mL, 5×10^4 cells/mL) and grown in a humidified incubator at 37°C and 5% CO_2 for 24 hours. According to the grouping in the section Effect of LBP in combination with DDP on the proliferation of A549 cells, the cells were incubated with test compounds for 24 hours. The culture medium was removed and the lysate was added at the ratio of 200 µL per well in the 6-well plate. The plate was blown on several times with a pipette so that the lysate was in full contact with the cells. Generally, the cell will be lysed after the lysate touches the cell for a few seconds, following which the cells are centrifuged at 10,000 rpm/min for 10 minutes and the supernatant aspirated for experiments. The total protein concentration was measured by BCA method. The protein was denatured by mixing 5 × sample buffer and protein sample at 1:4 (v/v). The proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%), and the bands were transferred on to polyvinylidene fluoride (PVDF) membrane. The bands were then sealed with 5% skimmed milk powder for 75 minutes, washed thrice with trimethylol aminomethane-hydrochloric acid-Tween 20 (TBST) buffer. Following this, the membrane was incubated overnight at 4°C with the primary antibody, and washed thrice using TBST. Next, the membrane was incubated using secondary antibody at room temperature and washed thrice with TBST. Finally, the chemiluminescence obtained was measured using an imaging system. The gray value of the protein band was measured using the Quantity One 4.4.0 software, and the relative expression of the protein was calculated using the formula,

$$\text{Relative protein expression} = \frac{\text{Gray level of protein band to be measured}}{\text{Gray scale of } \beta - \text{actin protein band}}$$

Data statistics

SPSS software (version 22.0) was used to analyze the data, and the results are expressed in terms of X ± S. One-way analysis of variance (ANOVA) was used to compare the data between the groups, and $\alpha = 0.05$ was used as the significance level. The experimental results are plotted with Origin 9.1 software.

RESULTS

Effect of DDP on the proliferation of A549 cells

Different concentrations of DDP showed varying degrees of inhibition of proliferation in CCK-8 method [Figure 1]. The rate of inhibition was in a dose-dependent manner, wherein an increase in the concentration of DDP decreased the rate of cell survival. Compared with the control group, the cell survival rate of each treatment group decreased significantly ($P < 0.01$). The IC_{50} of DDP was 11.90 mg/L.

Effect of LBP on the proliferation of A549 cells

As shown in Figure 2, LBP (>8 mg/L) significantly inhibited the proliferation of A549 cells in a dose-dependent manner as measured by CCK-8 method. The IC_{50} of LBP was 16.27 mg/L.

Effect of LBP in combination with DDP on the survival rate of A549 cells

As shown in Table 1, LBP and DDP showed a combined inhibitory effect on the proliferation of A549 cells. The combination of LBP and DDP based on their IC_{50} values in A549 cells (≥8 mg/L and 6 mg/L, respectively), the survival rate of A549 cells was around 50.1 ± 5.2%, which was the closest to 50%. In the subsequent experiment, if we choose

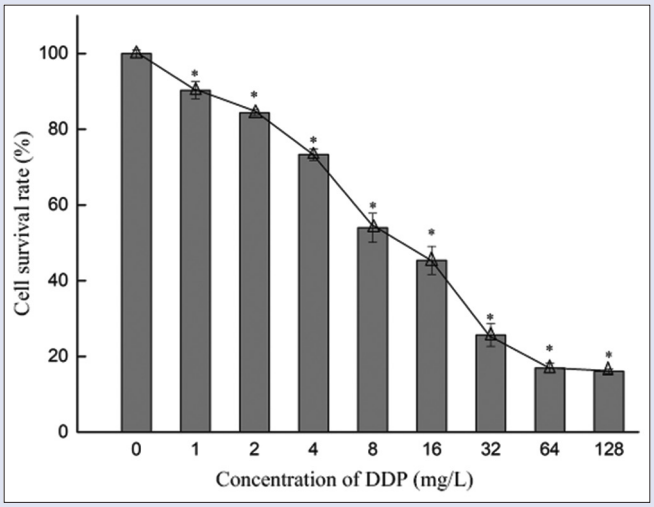

Figure 1: Effect of different concentrations of cisplatin (DDP) on the viability of A549 cells as measured by CCK-8 method ($n = 3$). *There was a significant difference between DDP and control group of cells ($P < 0.01$)

too high a dose of DDP and LBP to treat the cells, the cell survival rate will be too low, and there will be too many cell fragments in the later stage of flow cytometry. This will affect our test results, making cell damage difficult to detect. Therefore, 6 mg/L and 8 mg/L were chosen as the appropriate concentrations for subsequent experiments for DDP and LBP, respectively.

Effect of LBP combined with DDP on the activity of SOD, and levels of GSH and MDA in A549 cells

As shown in Figure 3, compared with the control group, DDP significantly decreased the activity of SOD and the content of GSH, and increased the content of MDA in the cells. LBP alone had no significant effect on the activity of SOD and the content of GSH and MDA in cells ($P > 0.05$). Compared with the control group, SOD activity and GSH content were significantly decreased, and MDA content was significantly increased in the LBP and DDP combined group ($P < 0.01$). Compared with the DDP group, there was no significant change of each index in the LBP and DDP combined group ($P > 0.05$).

Effect of LBP in combination with DDP on ROS content in A549 cells

As shown in Figure 4 and Table 2, compared with the control group, DDP alone significantly increased the ROS content in A549 cells ($P < 0.01$), whereas LBP alone significantly decreased the ROS content in A549 cells ($P < 0.01$). However, the combination of LBP and DDP did not cause any significant change in the intracellular ROS levels as compared with control group of cells ($P > 0.05$).

Effect of LBP combined with DDP on the apoptosis of A549 cells

The apoptosis rate of A549 cells was detected by Annexin V-FITC double staining method. According to the results, DDP significantly increased the apoptosis rate of A549 cells ($P < 0.01$). The apoptosis rate of A549 cells after incubating with LBP in combination with DDP was found to be significantly higher than that of DDP group ($P < 0.01$), which indicates that LBP promotes DDP-induced apoptosis in A549 cells [Table 3].

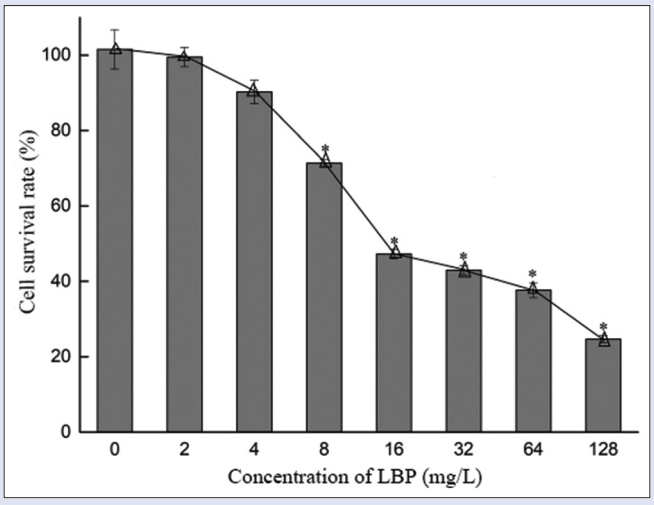

Figure 2: Effect of different concentrations of *Lycium barbarum* polysaccharide (LBP) on the viability of A549 cells as measured by CCK-8 method ($n = 3$). *There was a significant difference between cisplatin (DDP) and control group of cells ($P < 0.01$)

Effect of LBP combined with DDP on the expression of apoptosis-related proteins in A549 cells

In order to further explore the effect of LBP combined with DDP on apoptosis of A549 cells, the expressions of Bcl-2, Bax, and active caspase-3 in different groups were detected via Western blot. Figure 5

Table 1: Effect of LBP combined with DDP on the survival rate of A549 cells (n=3)

Concentration of LBP (mg/L)	Concentration of DDP (mg/L)		
	0	6	12
0	100.00±1.275	68.83±2.550	49.02±0.986
8	76.7±1.402	50.1±1.942	39.99±1.485
16	58.4±1.739	47.02±1.248	30.56±1.667

Abbreviations: LBP, *Lycium barbarum* polysaccharide; DDP, cisplatin

Table 2: Effect of LBP in combination with DDP on the content of ROS in A549 cells (X±S) (n=3)

Group	Relative ROS content
Control	1.000±0.032[a]
DDP (6 mg/L)	1.257±0.018[b]
LBP (8 mg/L)	0.854±0.063[c]
DDP (6 mg/L) + LBP (8 mg/L)	1.019±0.142[a]

Note: Same letters means no significant difference (P>0.05), but different letters mean significant difference (P<0.01). Abbreviations: LBP, *Lycium barbarum* polysaccharide; DDP, cisplatin; ROS, reactive oxygen species

Table 3: Effect of LBP combined with DDP on A549 cells apoptosis (n=3)

Group	Apoptosis rate (%)
Control	3.57±1.30[a]
DDP (6 mg/L)	28.36±3.47[b]
LBP (8 mg/L)	5.25±0.93[a]
DDP (6 mg/L) + LBP (8 mg/L)	45.55±6.04[d]

Note: Values with same superscripted letter indicate that there is no significant difference (P>0.05), but different letters mean significant difference (P<0.01). Abbreviations: LBP, *Lycium barbarum* polysaccharide; DDP, cisplatin; ROS, reactive oxygen species

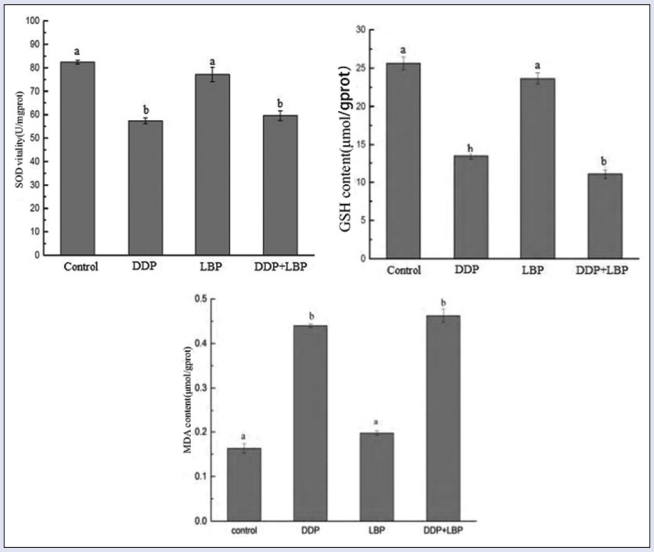

Figure 3: Effects of LBP combined with DDP on GSH and MDA content in A549 cells ($n = 3$). Same letter indicates no significant difference ($P > 0.05$), but different letters mean significant difference ($P < 0.01$)

Table 4: Relative expression of cell-associated proteins in different treatment groups (*n*=3)

Group	Relative expression of protein			
	Bcl-2	Bax	Bax/Bcl-2	Activated Caspase-3
Control	0.515±0.044[a]	0.263±0.0045[a]	0.482±0.0023[a]	0.553±0.036[a]
DDP (6 mg/L)	0.378±0.023[b]	0.454±0.0017[b]	1.201±0.029[b]	0.782±0.137[b]
LBP (8 mg/L)	0.540±0.026[a]	0.369±0.0118[c]	1.311±0.015[c]	0.592±0.053[a]
DDP (6 mg/L) + LBP (8 mg/L)	0.328±0.290[b]	0.706±0.007[d]	2.153±0.018[d]	1.141±0.127[c]

Note: Values with same superscripted letter indicate that there is no significant difference (P>0.05), but different letters mean significant difference (P<0.01).
Abbreviations: LBP, *Lycium barbarum* polysaccharide; DDP, cisplatin

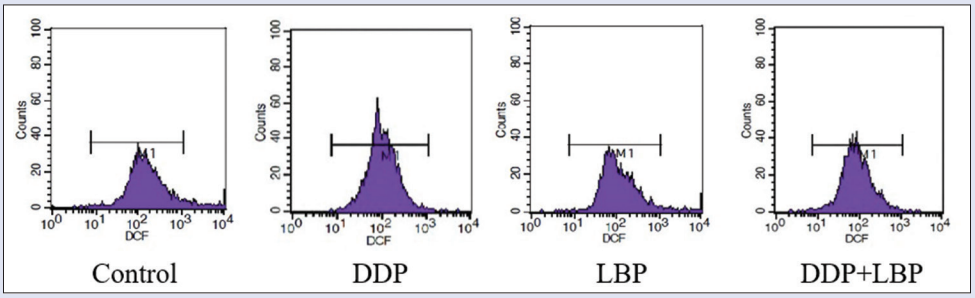

Figure 4: Effect of LBP combined with DDP on ROS content in A549 cells (*n* = 3). LBP, *Lycium barbarum* polysaccharide; DDP, cisplatin; ROS, reactive oxygen species

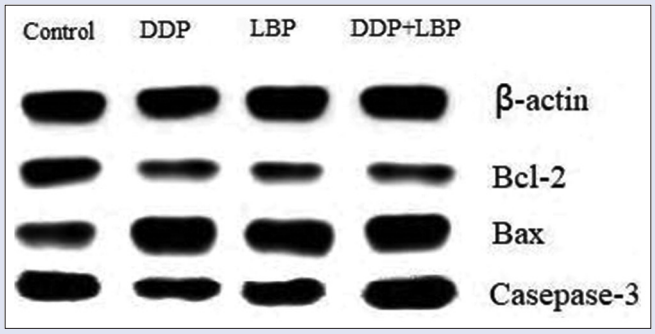

Figure 5: Effect of LBP combined with DDP on apoptosis-related protein expression in A549 cells (*n* = 3). LBP, *Lycium barbarum* polysaccharide; DDP, cisplatin

and Table 4 show the results of Western blot analysis. Compared with the control group, both DDP and LBP could significantly upregulate the expression of Bax and Bax/Bcl-2, and DDP could significantly reduce the expression of Bcl-2 and upregulate the expression of active caspase-3 (P < 0.01). The expression levels of Bcl-2 and active caspase-3 in the LBP-alone group were not significantly different from those in control group (P > 0.05). The expression of apoptosis-related proteins in LBP combined with DDP group was significantly different from that in the control group and LBP group (P < 0.01). Compared with DDP group, the expression levels of other proteins were significantly increased except for Bcl-2 (P < 0.01). The results showed that LBP combined with DDP may regulate the apoptosis of A549 cells by upregulating the expression of Bax and caspase-3, and the level of Bax/Bcl-2.

Effect of LBP combined with DDP on A549 cell cycle

The DNA content of cells in the G0/G1 phase of cell cycle is 2*N* (*N* represents the number of genomes, the same below), and the DNA content of cells in the G2/M phase contains a double genome and is 4*N*. Theoretically, the DNA content of cells in the S phase that are undergoing DNA replication should be between 2*N* and 4*N*. As shown in Figure 6 and Table 5, compared with the control group, the proportion of cells in G0/G1 phase in DDP group decreased significantly, whereas the proportion of cells in S phase increased significantly (P < 0.05). This indicates that DDP could block cells in S phase. Compared with the control group, the proportion of cells in G2/M phase in LBP group increased significantly, whereas the proportion of cells in S phase decreased significantly (P < 0.05). This indicates that LBP could block cells in G2/M phase. Compared with the DDP group, the proportion of cells in G2/M phase in LBP and DDP combined group increased significantly, whereas the proportion of cells in S phase decreased significantly (P < 0.05). Compared with the control group, the proportion of cells in G0/G1 phase in LBP and DDP combined group decreased significantly (P < 0.05). Our results showed that LBP in combination with DDP could not only form G2/M phase block, but also form S phase block, and finally induce apoptosis of A549 cells.

Effect of LBP combined with DDP on the expression of cycle-related proteins in A549 cells

In order to further prove the blocking effect of LBP combined with DDP on A549 cell cycle, the expression of cyclin D1, p-Rb, and CDK4 were detected by Western blot. As shown in Figure 7 and Table 6, compared with the control group, the protein expressions of p-Rb, CDK4, and cyclin D1 were significantly decreased after DDP treatment alone (P < 0.05). The expression levels of CDK 4, p-Rb, and cyclin D1 were significantly decreased after LBP treatment alone compared with the control group (P < 0.05). Compared with the DDP group, the expression levels of p-Rb and cyclin D1 were significantly increased (P < 0.05). In the case of DDP combined with LBP, the protein expressions of p-Rb, CDK4, and cyclin D1 were significantly decreased compared with the control group (P < 0.05), and the protein expressions of cyclin D1 and p-Rb were significantly decreased compared with the LBP group (P < 0.05). These results suggest that LBP combined with DDP mediate apoptosis via regulation of cyclin D1-CDK4-p-Rb pathway.

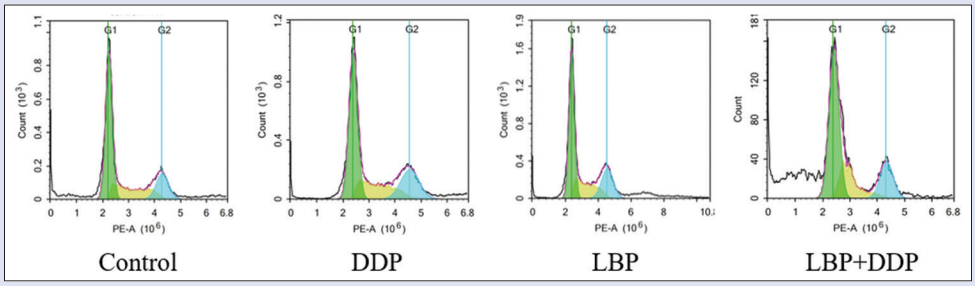

Figure 6: Effect of LBP and DDP on A549 cell cycles. LBP, *Lycium barbarum* polysaccharide; DDP, cisplatin

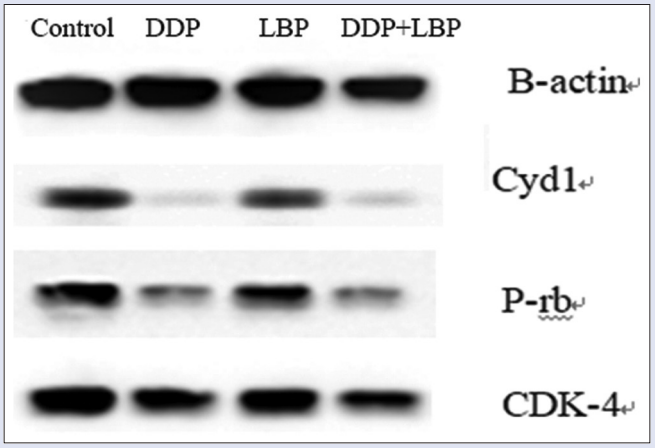

Figure 7: Effect of LBP combined with DDP on the relative expression of A549 cyclin. LBP, *Lycium barbarum* polysaccharide; DDP, cisplatin

Table 5: Effect of LBP and DDP on A549 cell cycles (*n*=3)

Group	G0/G1%	S%	G2/M%
Control	57.13±3.97[a]	25.65±4.31[a]	17.22±4.41[a]
DDP	49.96±5.26[b]	31.19±2.57[b]	18.83±4.32[a]
LBP	55.74±2.2[a]	18.63±4.12[c]	25.62±5.6[b]
DDP + LBP	50.96±6.56[b]	26.09±2 0.34[a]	22.94±4.73[b]

Note: Values with same superscripted letter indicate that there is no significant difference (P>0.05), but different letters mean significant difference (P<0.05). Abbreviations: LBP, *Lycium barbarum* polysaccharide; DDP, cisplatin; ROS, reactive oxygen species

Table 6: Effect of LBP combined with DDP on the relative expression of A549 cyclin (*n*=3)

Group	Relative expression of protein		
	CDK 4	p-Rb	Cyclin D1
Protein type			
Control	0.994±0.135[a]	0.704±0.248[a]	0.667±0.195[a]
DDP	0.757±0.249[b]	0.131±0.102[b]	0.036±0.213[b]
LBP	0.701±0.074[b]	0.519±0.013[c]	0.392±0.021[c]
DDP + LBP	0.732±0.179[b]	0.143±0.213[b]	0.038±0.204[b]

Note: Values with same superscripted letter indicate that there is no significant difference (P>0.05), but different letters mean significant difference (P<0.01). Abbreviations: LBP, *Lycium barbarum* polysaccharide; DDP, cisplatin; ROS, reactive oxygen species

DISCUSSION

DDP is effective in the treatment of lung cancer. The anti-cancer activity of DDP is determined by the chloride ion concentration in the environment. Cisplatin alone has low activity and cannot play its role well; so cisplatin is partially hydrolyzed after it enters the cell, and the intracellular water molecules attack the chlorine ligand, resulting in [P $(NH_3)_2(H_2O)$ Cl] and [Pt $(NH_3)_2(H_2O)_2$]. From the point of view of kinetics, the hydrolysis of cisplatin to [Pt $(NH3)_2(H2O)$ Cl] is a second-order reaction. The reaction rate depends on the concentration of chloride ion and cisplatin. The higher the concentration of chloride ion in the environment, the smaller the reaction rate. In blood and extracellular tissue fluid, the concentration of chloride ions is high, so DDP is relatively stable. When it enters the cell fluid, the concentration of chloride ion is very low, and the two chloride ions in DDP will be replaced by water to form hydrate. This hydrate can easily cross-link with DNA, which interferes with DNA replication and transcription, leading to apoptosis.[3] As tumor cells are actively divided, they become more sensitive to the DNA damage caused by DDP than normal cells. The anti-cancer activity of DDP is mainly due to the formation of DNA adducts.[10] In addition, mitochondrial DNA can easily mutate and be cross-linked by DDP, causing damage to the cells. The nucleophilic amino group contained in DDP can also interact with water molecules to produce a large number of free radicals, which further damage mitochondria, eventually leading to cell death.[11] A previous study has shown that the bioavailability of DDP is low, about 10%–35%.[12] For adults, 10–20 mg/day of DDP (1.05–3.50 mg/L) dissolved in 200 mL of normal saline is the recommended dosage (bioavailability of 35%). In this study, the injury model of A549 cells was established by DDP. The results showed that DDP at a concentration of ≥1 mg/L could significantly inhibit the proliferation of A549 cells in a dose-dependent manner. DDP at 6 mg/L concentration could induce apoptosis of A549 cells. However, DDP can cause serious side effects on normal tissues (such as renal toxicity, blood toxicity, and ototoxicity),[13–15] which limit the long-term use of DDP in patients with cancer. Therefore, it has become an important task to find a highly effective and low-toxicity tumor chemotherapy sensitizer to enhance the efficacy of DDP chemotherapy and improve the quality of life of patients with cancer.

In recent years, natural drugs have played an important role in the treatment of cancer. At present, many active ingredients extracted from natural drugs, such as astragalus polysaccharide, ganoderma polysaccharide, and matrine, have been found to have good antitumor effects.[16–18] LBP is an active polysaccharide extracted from the fruit of *L. barbarum*. Studies have shown that LBP has good antitumor activity.[7] LBP has been used in traditional Chinese medicine in combination chemotherapy.[19] Our results suggest that LBP (8 mg/L) alone significantly inhibited the growth and proliferation of A549 cells, and its bioavailability was about 36.01%. The recommended daily intake of LBP for cancer treatment is 1,600–2,400 mg (12–18 mg/L). LBP combined with DDP can significantly reduce the survival rate of A549 cells, indicating that LBP can enhance the inhibitory effect of DDP on the growth of A549 cells.

ROS can be involved in regulating the activity of a variety of molecules and signal transduction pathways in cells. Compared with normal cells, the production of ROS in tumor cells is significantly increased, mainly due to the influence of tumor genes, mitochondrial function variation, and other factors, which put the cells in a higher state of oxidative stress.[20] According to Finkel *et al.*,[21] a small amount of ROS can act as a signaling molecule to mediate signal transduction pathways, and play a protective role on cells by participating in inflammatory and immune responses. However, excessive formation of ROS can cause lipid peroxidation and form the peroxide product MDA, which will lead to DNA damage or apoptosis of cells. The results showed that DDP alone could decrease the content of GSH and SOD activity in A549 cells, and the content of MDA and ROS in A549 cells were significantly increased ($P < 0.01$). DDP induced oxidative stress in A549 cells leading to apoptosis. Under normal physiological conditions, intracellular ROS is regulated by the antioxidant system to maintain a balanced state. However, DDP promotes the oxidative stress response of cells by damaging the electron transport chain of mitochondrial respiration, leading to the accumulation of intracellular ROS.[11,22,23] In the body, the antioxidant system (SOD, GSH, etc.) can remove the excess ROS to maintain the balance of the REDOX state. SOD is a highly efficient ROS scavenging agent, which exists in almost all biological cells. SOD catalyzes the transformation of superoxide anion radical ($O_2^{-\bullet}$) into harmless H_2O by interacting with peroxidase and oxidase, thereby protecting cells. As an important antioxidant and free radical scavenger, GSH has a powerful detoxification effect. Therefore, both SOD and GSH can be used as important indexes of the antioxidant capacity of cells.[24] The experimental data showed that compared with DDP alone, LBP combined with DDP significantly reduced ROS content ($P < 0.01$), whereas GSH and MDA content and SOD activity had no significant changes ($P > 0.05$). DDP can inhibit the activity of antioxidant enzymes in cells, thereby causing imbalance in the redox state and promote the lipid peroxidation process and apoptosis. However, LBP combined with DDP had no significant effect on the imbalance in the redox system caused by DDP, but it could effectively eliminate ROS produced by A549 cells. He *et al.*[25] reported that ROS inhibitors could reduce the anti-cancer activity of DDP. Results of apoptosis study showed that the apoptosis rate of A549 cells increased from 3.57% to 28.36% by DDP alone, which increased to 45.55% after combining it with LBP, suggesting that LBP can significantly enhance the pro-apoptotic effect of DDP on A549 cells. However, oxidative stress is not the primary mechanism of action of LBP in promoting DDP-induced apoptosis.

Regulation of cell cycle is an important concept in cell proliferation and apoptosis. The cell cycle can be divided into three stages: G0/G1, S, and G2/M, in which RNA replication and related protein synthesis are carried out in G1 and G2 phases, whereas DNA replication is carried out in S phase.[26] S-phase arrest can provide sufficient time for the repair of damaged DNA in cells. When the damaged DNA exceeds the repair or tolerance limit of the cell, the functional sensitivity of the checkpoint in S phase will be reduced, and eventually, cell apoptosis or death will result from the inability of intracellular DNA damage to be effectively repaired. Previous studies have shown that DDP can arrest cells in S phase, and its toxicity can be increased when S-phase arrest is removed with other drugs.[27,28] Therefore, it is hypothesized that there is a certain relationship between S-phase arrest and the drug resistance of cancer cells to DDP. The results of this study showed that DDP alone could induce the S-phase arrest of A549 cells, whereas LBP alone could induce the G2/M phase arrest of A549 cells, indicating that the damage of LBP to cancer cells might be mainly reflected after the completion of DNA replication. The combination of DDP and LBP can block A549 cells in G2/M phase and S phase. It is suggested that the combined blocking effect of DDP and LBP on cell cycle further inhibits cell proliferation.

The regulation of cell cycle can be divided into exogenous regulation and endogenous regulation. Exogenous regulation is mainly caused by external stimuli. In endogenous regulation, cyclin and cyclin-dependent kinase (CDK) play a key role. CDK is a kind of serine/threonine protein kinase, and its family includes CDK1 ~8, of which CDK4 is the core regulator of cell cycle and the key protein kinase for the occurrence and development of most malignant tumors.[29] Therefore, it has become one of the important targets of antitumor drugs. It combines with cyclin and regulates the transformation of each phase of the cell cycle. Cyclin D1 is thought to be a proto-oncogene protein that is mutated or highly expressed in a variety of human cancer cells.[30] Furthermore, Rb is a protein expressed by tumor suppression genes, and the activity of Rb protein is mainly determined by its phosphorylation and dephosphorylation levels.[31] Cyclin D1 can combine with CDK4 to form a complex, which can phosphorylate Rb protein to form p-Rb, so as to achieve the promotion and transformation of different phases of the cell cycle.[32,33] The results of this study showed that DDP could significantly reduce the protein expression levels of p-Rb, CDK4, and cyclin D1 ($P < 0.05$), causing S-phase arrest of cells. Compared with the control group, LBP alone significantly reduced the protein expression levels of cyclin D1, CDK4, and p-Rb. However, compared with DDP alone, the protein levels of p-Rb and cyclin D1 were significantly increased ($P < 0.05$), and the cells produced G2/M-phase arrest. Compared with the control group, the protein expressions of p-Rb, CDK4 and cyclin D1 in LBP and DDP combined group were significantly decreased ($P < 0.05$). Compared with LBP group, the expression of cyclin D1 and p-Rb proteins in LBP and DDP combined group was also significantly decreased ($P < 0.05$). Cells form G2/M-phase and S-phase arrests. It is suggested that the inhibition of A549 cells by LBP and DDP alone or together is related to the cell cycle regulation mediated by cyclin-D1-CDK4-p-Rb pathway, and the specific mechanism remains to be further studied.

Apoptosis is a programmed cell death, which is usually accompanied by the rupture of the mitochondrial membrane and the release of cytochrome C from the mitochondria to the cytoplasm.[34] Bcl-2 is an anti-apoptotic protein that plays a major role in regulating apoptosis in the feedback loop system with caspase family proteins by changing the permeability of the mitochondrial membrane.[35,36] Bax protein can promote apoptosis and is an important part of the apoptotic pathway. According to the literature, downregulation in the expression of Bcl-2 and an upregulation in the expression of Bax will increase the permeability of mitochondrial membrane, which promotes the release of apoptosis-activating factors such as cytochrome C, and further enhancing the activation of caspase-3 to induce apoptosis.[37] Kumar *et al.*[38] showed that caffeic acid in combination with DDP upregulated the expression of caspase-3 and Bcl-2, thus promoting the apoptosis of human cervical cancer cells. Liu *et al.*[39] used single-cell analysis to study the distribution kinetics of Bax in the process of DDP-induced apoptosis of human lung adenocarcinoma cells. These results also showed that LBP and DDP alone downregulated the expression of Bcl-2 and upregulated the expression of Bax and active caspase-3 in A549 cells, suggesting that LBP and DDP can induce apoptosis of A549 cells through pro-mitochondrial release of apoptotic factors. In addition, the ratio of Bax/Bcl-2 is crucial for reflecting drug-induced apoptosis of cancer cells, and its significance is superior to the expression of Bcl-2. LBP in combination with DDP significantly upregulated the expression of active caspase-3 and the levels of Bax/Bcl-2, following which the mitochondrial permeability transition pore are opened. Cytochrome C, caspase-9, and other pro-apoptotic proteins flow out, thereby promoting cellular apoptosis. LBP in combination with DDP could promote apoptosis of A549 cells, which was related to the upregulation of Bax, downregulation of Bcl-2, and activation of caspase-3 expression.

CONCLUSION

In summary, LBP can be used in combination with DDP to inhibit the growth of human lung adenocarcinoma A549 cells, and it can enhance DDP-induced apoptosis of A549 cells. The combined effect is related to the regulation of cell cycle mediated by cyclin D1-CDK4-p-Rb pathway and the regulation of the expression of apoptosis-related proteins in cells, whereas the antioxidant capacity of LBP has no significant effect on the apoptosis-promoting effect of DDP. The results of this study might provide some reference for the combination of antitumor natural products in the future.

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Conflicts of interest

There are no conflicts of interest.

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