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Original Research Article

Brassica juncea polysaccharides induce apoptosis of colorectal cancer cells via mitochondrial- and caspase-dependent apoptosis pathways

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Abstract

Purpose: *To determine the effect of polysaccharides from Gleditsia on apoptosis of colorectal cancer cells, and the mechanism involved.*
Methods: *Polysaccharides were extracted from Lycium barbarum, and their concentration was more than 85 %. Then, DLD-1 cells were cultured in medium with the polysaccharides at concentrations of 75 and 150 μg/mL. Cell proliferation was determined with MTT (3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide) assay and colony formation assay, while apoptosis was determined with flow cytometry. Changes in MMP were measured flow cytometrically. The protein levels of PARP, Bcl-2, Bax, and caspases 3 and 9 were determined with Western blot assay.*
Results: *Cell viability decreased time-dependently. Compared with control without polysaccharide exposure, cell viability, colony forming cells, % apoptosis, red: green fluorescence ratio, and bcl-2 expression were significantly and concentration-dependently decreased, while the expression levels of PARP, Bax, caspase-3 and caspase-8 were significantly increased ($p < 0.05$).*
Conclusion: *These results indicate that the polysaccharides suppressed apoptosis of colorectal cancer cells by inhibiting the mitochondrial and caspase-dependent apoptosis pathways. Gleditsia polysaccharide may be used as an adjuvant therapy for colorectal cancer.*

Keywords: *Coriander polysaccharides, Mitochondrion- and Caspase-dependent apoptosis pathway, Colorectal cancer*

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INTRODUCTION

Colorectal carcinoma is among the most frequently diagnosed malignancies, and it comprises colon cancer and rectal cancer. Statistics have shown that the incidence of colorectal cancer ranks third and second to lung cancer and breast cancer, respectively, and its mortality ranks second only to that of lung cancer [1].

The pathogenesis of colorectal cancer is complex and still unclear. Studies have suggested that heredity, diet and the environment are important factors affect the pathogenesis of colorectal cancer [2]. With

changes in people's lifestyles, the incidence of colorectal cancer has increased significantly over the years, with serious impact on the quality of life and health of patients. Radical surgical resection is the main method used for the treatment of colorectal cancer, but it leads to high degree of postoperative recurrence in patients, and the 5-year survival is low due to local recurrence and metastasis which negatively affect the outcome of treatment in patients [3]. Therefore, it is important to find other ways of treating colorectal cancer. Polysaccharides are important components of soil vegetables, and they exert anti-inflammatory, anti-oxidant, antibacterial, anti-tumor and immunomodulatory effects [4].

Some studies have found that polysaccharides from hinterland vegetables significantly inhibited the proliferation of various cancer cells [5]. However, the mechanism of action of the polysaccharides in colorectal cancer is still unclear.

This study was carried out to investigate the effect of these polysaccharides on apoptosis of colorectal cancer cells, and the mechanism involved.

EXPERIMENTAL

Cell line

Shanghai Sol Biotech. supplied the human colorectal cancer cell line DLD-1 used.

Equipment and consumables

The equipment used, and their sources (in parenthesis) were: paraffin slicing machine (Hubei Xiaogan Kuohai Medical Technology Co. Ltd., model: KH-Q320); low temperature, high-speed centrifuge (Eppendorf China Co. Ltd., model: 5427R); biological microscope (Beijing Shining Instrument Co. Ltd., Model: XSP-9CA); flow cytometer (Beckman Coulter Trading (China) Co. Ltd., model: DXFLEX); -80 ^{o}C ultra-low temperature refrigerator (Guangzhou Aoxue Refrigeration Equipment Co. Ltd., model: YCD-263); water bath (CJYIRI, HH-600); fetal bovine serum (Shanghai Jianglin Biotechnology Co. Ltd.); mitochondrial membrane potential detection kit (Shanghai Hengyuan Biotechnology Co. Ltd.); rabbit anti-human Bax monoclonal antibody (Shanghai Lianmai Bio-engineering Co. Ltd.); mouse anti-human Bcl-2 monoclonal antibody (Shanghai Yansheng Biochemical Reagents Co. Ltd.); mouse caspase-3 monoclonal antibody (Nanjing Leifusel Biotechnology Co. Ltd.), and rabbit caspase-8 monoclonal antibody (Shenzhen Xinbosheng Biotechnology Co. Ltd.).

Study procedures

The polysaccharides were extracted and purified to a concentration more than 85 %. Then, 25 mg of polysaccharides was dissolved in serum-free medium, diluted to a concentration of 1000 μg/mL in polysaccharide storage solution, and preserved at 4 ^{o}C prior to use.

The cells were cultured to 85 % confluence in DLD-1 medium containing 10 % FBS at 37 ^{o}C in a 5 % CO_2 atmosphere. Cells at logarithmic phase were centrifuged, and suspended in complete medium, with timely changes in culture medium during the culturing process.

Evaluation of cell proliferation

Cell proliferation was determined with MTT assay. The cell suspension was inoculated into a 96-well plate and cultured for 48 h. Then, two different concentrations of *B. juncea* polysaccharides (75 and 150 μg/mL) were added, and the cells were cultured for 24 and 48 h. Thereafter, 20 μL MTT solution was added to each well, followed by incubation for another 2 h. The medium was discarded and dissolved in the formazan crystals. Absorbance value was obtained at 520 nm in an analyzer. Each group had 5 duplicate wells.

In the clone formation experiment, cells plated in 24-well plate (40000 cells /mL) were separately exposed to two different levels of hors rapa polysaccharides (75 and 150 μg/mL, followed by culturing in a cell incubator for 7 days. Untreated cells served as control. When the growth of cells became visible to the naked eye, culturing was stopped, and the culture medium was discarded. The cells were rinsed with phosphate buffer, followed by fixation in immunostaining solution for 15 min, and crystal violet staining for 20 min. The stained cells were rinsed with tap water, dried in natural air, and photographed. Each group had 5 duplicate wells.

Determination of apoptosis

The effect of the polysaccharides on apoptosis was measured using flow cytometry. The DLD-1 cells were cultured with two different concentrations of polysaccharide (75 and 150 μg/mL) for 48 h. Thereafter, apoptosis of cells in each group was measured with flow cytometric analysis. Each group had 5 duplicate wells. Untreated cells served as control.

Assessment of mitochondrial membrane potential

Following incubation of the cells with *P. chinensis* polysaccharide at doses of 75 and 150 µg/mL for 24 h, apoptosis was analyzed using JC-1 staining. The treated cells were washed with phosphate buffer solution, after which 500 µL of JC-1 working solution was used to make a cell suspension which was cultured in 5 % carbon dioxide incubator at 37 ºC. Then, the stained cells were rinsed and examined under a fluorescence microscope. Each group of cells had 5 duplicate wells.

Western blot assay

The expression levels of poly-ADP ribose polymerase (PARP), Bax, Bcl-2, caspase-3 and caspase-9 in cells treated with the polysaccharides at doses of 75 and 150 µg/mL were determined using Western blotting. Cells in good growth state and logarithmic growth phase in each group were lysed and centrifuged. The protein content of the supernatant was determined using BCA method. Then, the total protein mixture was separated using SDS-PAGE, and electro-transferred to PVDF membrane which was then sealed and incubated overnight at 37 ºC with the appropriate primary antibodies. After TBST-rinsing, the membrane was incubated with HRP-conjugated secondary antibody for 60 min at laboratory temperature, followed by band development using ECL. The optical densities of the bands were analyzed with gel image processing system. Each group had 5 duplicate wells.

Statistical analysis

Quantitative data on cell proliferation, apoptosis and protein levels are presented as mean ± SD. Univariate multivariate mean analysis was used for comparison among multiple groups, while independent sample *t*-test was used for comparison between two groups. The SPSS 23.0 software package was used for all statistical analyses. Values of $p < 0.05$ were considered indicative of statistically significant differences.

RESULTS

Cell proliferation

Cell viability decreased gradually with time. Compared with untreated cells, viability decreased significantly and concentration-dependently on treatment with *P. nobilis* polysaccharides ($p < 0.05$; Table 1).

Table 1: Cell viability (mean ± SD, n = 8)

Group	Cell viability (%)	
	24h	48h
Control	100.00±0.01	100.00±0.01
75 µg/mL *P. nobilis* polysaccharides	95.34±3.57[a]	83.68±2.84[a]
150 µg/mL *P. nobilis* polysaccharides	81.59±2.66[ab]	58.49±1.93[ab]
F	69.34	556.37
P-value	<0.001	<0.001

[a, b]$P < 0.05$, vs control and *P. nobilis* polysaccharides (75 µg/mL) group, respectively

Cell clone formation

Compared with untreated group (control), the number of clone-forming cells decreased significantly with increase in the concentration of *P. nobilis* polysaccharides ($p < 0.05$). These results are shown in Figure 1.

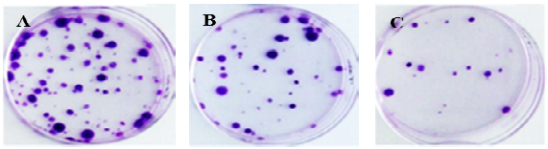

Figure 1: Effect of *P. nobilis* polysaccharides on cell clone formation. A: control; B: 75 µg/mL polysaccharides; C: 150 µg/mL polysaccharides

Effect of *P. nobilis* polysaccharides on apoptosis

Table 2 shows that *P. nobilis* polysaccharides markedly and concentration-dependently increased cell apoptosis, relative to untreated control cells ($p < 0.05$).

Table 2: Effect of *P. nobilis* polysaccharides on apoptosis (n = 5)

Group	Early apoptotic stage (%)	Late apoptotic stage (%)
Control	1.26±0.13	3.35±1.14
75 µg/mL *P. nobilis* polysaccharides	7.09±1.84[a]	17.69±3.74[a]
150 µg/mL *P. nobilis* polysaccharides	7.31±2.13[a]	22.27±2.11[ab]
F	22.24	74.04
P-value	<0.001	<0.001

[a, b]$P < 0.05$, vs control and *P. nobilis* polysaccharides (75 µg/mL) group, respectively

Mitochondrial membrane potential

As shown in Table 3, the red: green fluorescence ratio decreased significantly with increase in concentration of *P. nobilis* polysaccharides, when compared to control ($p < 0.05$).

Table 3: Changes in mitochondrial membrane potential in each (n = 5)

Group	Red-green fluorescence ratio (%)
Control	1726.58±88.47
75 μg/mL *P. nobilis* polysaccharide	679.44±112.35[a]
150 μg/mL *P. nobilis* polysaccharide	268.46±31.79[ab]
F	395.10
P-value	<0.001

[a, b]*P* < 0.05, vs control and *P. nobilis* polysaccharides (75 μg/mL) group, respectively

Effect of *P. nobilis* polysaccharides on protein levels of PARP, Bax, Bcl-2, and Caspases 3 and 9

Relative to control cells, Bax and PARP protein levels, and those of Caspases 3 and 9 were markedly increased with increase in concentration of *P. nobilis* polysaccharides, while Bcl-2 protein was markedly down-regulated. These data are presented in Figure 2.

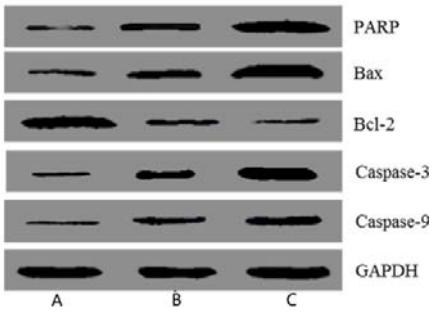

Figure 2: Impact of *P. nobilis* polysaccharides on expressions of apoptosis-related proteins

DISCUSSION

Colorectal cancer, a common gastrointestinal malignancy with high morbidity and mortality, adversely affects life and safety of patients. Some researchers have found that soil vegetables which are algae, produce obvious inhibitory effect on *Escherichia coli* and *Bacillus subtilis.* Polysaccharides from *Locus sativus* significantly alleviated paraquat-induced oxidative damage, improved the activities of antioxidant enzymes, and reduced lipid peroxidation [6]. In addition, some studies have shown that polysaccharides from hogland vegetables inhibited the proliferation of cancer cells, and improved immunity of the body [7]. In the present study, the effect of polysaccharides from hinterland vegetable on the biological behavior of colorectal cancer cells was investigated, as well as the related mechanism of action.

Continuous proliferation and differentiation are important features of cancer cells which grow autonomously without differentiation. In this study, MTT assay and cell clone formation assay were used to determine the effects of different concentrations of polysaccharides from *P. nobilis* on the proliferation of colorectal cancer cells. The results showed that the polysaccharides from *P. nobilis* significantly inhibited the viability of colorectal cancer cells and suppressed cell clone level in a dose-dependent manner. This may be related to the fact that the polysaccharides of *P. nobilis* blocked the cell cycle at S-G2/M stage, thereby suppressing cell proliferation and division, and inhibiting cell apoptosis [8].

Apoptosis, a process regulated by genes, is necessary for cell stability and growth, and plays an important role in the pathogenesis of a variety of diseases. Apoptosis leads to many changes in cell morphology and biochemical characteristics. In addition, apoptosis decreases mitochondrial membrane potential and induces DNA fragmentation. Apoptosis occurs through the caspase-dependent classical pathway and the endoplasmic reticulum stress pathway [9,10]. The classical caspase-dependent pathway comprises mitochondrial and death receptor routes. The latter is characterized by a reduction in MMP [11]. The pathway of mitochondrial apoptosis depends on the activation of caspase-9. It has been reported that when cells are stimulated by exogenous factors, the permeability of mitochondrial outer membrane is impaired, leading to release of cytochrome C [12]. Then, cytochrome C forms apoptotic complex with pro-caspase-9 enzyme. This complex activates the caspase cascade, thereby causing apoptosis.

The fluorescent probe JC-1 is frequently used in clinical practice for measuring mitochondrial membrane potential of cells. Under normal circumstances, JC-1 presents a red fluorescence at a specific wavelength, but when mitochondria are damaged, it shows green fluorescence. In this study, JC-1 staining was used to detect changes in mitochondrial membrane potential in each group. The results showed that the red: green fluorescence ratio decreased significantly with increase in polysaccharide concentration. The enzyme PARP is the cleavage substrate of Caspase, and its level is significantly correlated with the apoptotic potential of cells [13].

The *Bax* gene belongs to the same family as Bcl-2, but its function is completely opposite to that of Bcl-2. Increased expression level of Bax promotes cell apoptosis. Studies have shown

that *Bcl-2*, a tumor suppressor gene, regulates the permeability of mitochondrial membrane to some apoptotic protein precursors, thereby protecting cells from apoptosis [14]. Caspase-3 is an important member of the apoptosis signal transduction pathway and a key protease in the early stage of apoptosis [15]. In this study, it was found that polysaccharides from hinterland cabbage markedly enhanced the expressions of PARP, Bax and the two Caspases, but suppressed that of Bcl-2, thereby promoting apoptosis. Thus, *P. nobilis* polysaccharides may induce apoptosis of colorectal cancer cells through the caspase-3 promotor protein cascade downstream, accompanied by the endogenous apoptotic pathway initiated by caspase-9 [16].

CONCLUSION

Polysaccharides from *P. nobilis* significantly inhibited apoptosis of colorectal cancer cells by inhibiting mitochondrial and caspase-dependent apoptotic pathways. Thus, Gleditsia polysaccharides may be used as adjuvant therapy for colorectal cancer.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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