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ORIGINAL ARTICLE

Silibinin promotes the apoptosis of gastric cancer BGC823 cells through caspase pathway

Runfang Li[1], Jianmin Yu[2], Chengjun Wang[3]

[1]Department of General Medicine, Chiping People`s Hospital, Liaocheng, China; [2]Department of Radiology, Weifang People`s Hospital, Weifang, China; [3]Department of Gastroenterology, Weifang People`s Hospital, Weifang, China

Summary

Purpose: *To investigate the effects of silibinin, a natural compound, on the proliferation and apoptosis of BGC-823 gastric cancer cell line and to figure out the relevant pathways.*

Methods: *BGC-823 gastric cancer cells were treated with silibinin at various concentrations (0,25,50,100,150 and 200μM). Zero μM was the control group and the other concentrations were defined as the experimental groups. The effects of silibinin on the proliferation of BGC-823 gastric cancer cells were explored through methylthiazolyldiphenyltetrazolium bromide (MTT). Silibinin's toxic effects were detected through determination of the concentration of lactic dehydrogenase (LDH). Flow cytometry was performed to explore the effects of silibinin on apoptosis of these cells. Western blotting was conducted to study the relevant pathways acting on the BGC-823 cells.*

Results: *MTT assay showed that with the increase in silibinin concentration and extension of exposure time, the inhibitory effect silibinin on cell proliferation was enhanced in an obvious time-dosage pattern. The results of LDH detection showed that the toxicity of silibinin to cells was enhanced in an obvious time-dosage pattern with the increase in drug concentration and extension of exposure time. Flow cytometry revealed that with the increase in drug concentration, gradual increase in the proportion of early and late of apoptotic cells took place, and the comparison between the experimental and the control group showed that the difference had statistical significance. Western blotting indicated that silibinin could upregulate the expression of mitochondrial apoptosis-associated proteins, and the difference in comparison with the control group had statistical significance.*

Conclusion: *Silibinin can inhibit the proliferation of BGC-823 gastric cancer cells, and such an inhibitory effect is time- and concentration-dependent. Additionally, silibinin can promote the apoptosis of BGC-823 gastric cancer cells, which may be realized through mitochondrial apoptosis.*

Key words: *apoptosis, caspase 3, gastric cancer BGC-823 cell line, mitochondrial apoptosis, silibinin*

Introduction

Gastric cancer is one of the common malignant tumors of the digestive system, and its treatment methods include surgery, chemotherapy, radiotherapy, molecular targeting therapy and bio-immunotherapy [1]. Systemic therapies are an integral part in the treatment of gastric cancer and natural compounds included in the traditional Chinese Medicines (TCM) are quite attractive as they possess strong biological activities, low toxicity and only few side-effects. TCM has now become a major method for the treatment of tumors and research of this antitumor activities and relevant mechanisms has been one of the research subjects among Chinese researchers [2,3].

Currently, the major pharmacological effect of chemotherapeutic drugs in the treatment of gas-

Correspondence to: Chengjun Wang, MD. Department of Gastroenterology, Weifang People's Hospital, 151 Guangwen Street, Kuiwen District, 261041 Weifang, Shandong, China.
Tel: +86 0182 62634, E-mail: 413609551@qq.com
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tric cancer is to induce cell apoptosis [4], which can be realized through mitochondrial and non-mitochondrial pathways [5]. Mitochondrial apoptosis pathway mainly refers that the entrance of Bax from cytoplasm to mitochondria and the release of cytochrome C from mitochondria to cytoplasm can induce a cascade of apoptotic events [6].

Silibinin is the major active ingredient in silymarin (flavonoid compound), and its content is highest [7]. Analyses have shown that silibinin and its isomers occupy almost 90% of silymarin [8]. Pharmacological studies suggest that silibinin is characterized by various features, such as anti-lipid peroxidation effect, elimination of free radicals, inhibiting the activity of lipoxygenase, maintaining stability of cell membrane, and accelerating the regeneration of hepatic cells [9]. In the last decade, *in vitro* and animal experiments have also revealed the antitumor effect of silibinin. According to some authors, silibinin exhibits strong inhibitory effects on a variety of epithelium-originated tumors, including prostate cancer, colorectal cancer, bladder cancer and lung cancer [10], implying thus that silibinin has a promising prospect in application for gastric cancer. It has been established that silibinin can inhibit the growth, invasion and metastasis of some tumor cells [11], but its impact on BGC-823 gastric cancer cells and the relevant mechanism remain unknown.

In this study, we investigated for the first time the effects of silibinin on the proliferation and apoptosis of BGC-823 gastric cancer cell line, which served as the experimental basis for in-depth research on the antitumor mechanism of silibinin.

Methods

Cells and reagents

BGC-823 gastric cancer cell line was purchased from Shanghai Cell Bank of China; silibinin was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Bax, Cyto C, Caspase 3, cleaved-Caspase 3 were purchased from Cell Signaling Technology (Danvers, MA, USA) and the secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Flow cytometry kit, MTT kit and LDH kit were purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China).

Cell culture

BGC-823 human gastric cancer cells were cultured in the high-sugar DMEM culture medium containing 10% FBS in an incubator (37°C, saturated humidity and 5% CO_2). Initially, cells were in monolayer growth against the wall, and when the bottom of the culture flask was covered by cells (generally about 2 to 3 days), the cell density was adjusted to 5×10^5/mL for digestion using 0.25% trypsin and passage under the septic environment.

MTT assay

Cell suspension was prepared through digesting the cells collected during the logarithmic growth phase, and the concentration was adjusted to 1×10^5/mL. Then, cells were inoculated onto the 96-well plate (100 µL/well). In this study, 3 replicate wells were set as control. Through culture overnight after inoculation, the cells were examined by microscope to affirm that their growth was good against the wall. Thereafter, silibinin, that had been diluted, was added into the cell culture to adjust the final concentrations to 0 µM, 25 µM, 50 µM, 100 µM, 150 µM and 200 µM. 0 µM group was set as the control group for high-sugar DMEM. Cells were then cultured for 24 and 48 hrs in an incubator (37°C, saturated humidity and 5% CO_2). At 4 hrs before the end of culture, 20 µL MTT was added into each well for culture at 37°C for 4 hrs. Then, the supernatant was carefully drawn, and 150 µL dimethylsulfoxide (DMSO) was added into each well followed by vibration for mixing for 10 min. The sediment was dissolved using formaldehyde. The optical density (OD) at 570 nm wavelength was determined using microplate reader. This experiment was repeated for 3 times. Inhibition of proliferation was calculated using the following formula: Inhibition rate = OD value in drug administration group/ OD value in control group ×100%.

LDH detection

Cell suspension was prepared through digesting the cells collected during the logarithmic growth phase, and the concentration was adjusted to 1×10^5/mL. Then, cells were inoculated onto the 96-well plate (100 µL/well). In this study, 3 replicate wells were set as control. After the cell growth against the wall was observed, silibinin that had been diluted was added into the cell culture to adjust the final concentrations to 0 µM, 25 µM, 50 µM, 100 µM, 150 µM and 200 µM. The culture plate was then placed into an incubator for 24 hrs of culture (37°C and 5% CO_2). Then, the cells were grouped, and 20 µL of supernatant was drawn from each well. In the extracted supernatant, the corresponding reagents were added according to the instruction of kit and mixed well. After the supernatant was placed for 3 min at room temperature, bi-distilled water was placed into a cuvette for zero adjustment at 440 nm wavelength, and the OD values were determined in the microplate reader. Definition of unit: 1 unit for 1 gmol pyruvic acid generated from the reaction system after 15 min reaction of 1000 mL culture medium and matrix at 37°C. LDH content in the culture medium was calculated according to the instructions of the LDH kit.

Detecting through flow cytometry

BGC-823 cells were inoculated into a 6-well plate at 5×10^5/mL concentration overnight, with silibinin in final concentrations of 0 μM, 50 μM, 100 μM and 200 μM, and cells were cultured in an incubator (37°C, saturated humidity and 5% CO_2) for 24 hrs, followed by digestion using trypsin. Harvested cells were centrifuged at 4°C for 4 min at 1000 rpm/min for collecting the cells and removing the medium. After cells that were collected after centrifugation were rinsed twice using cold phosphate buffer saline (PBS), cell suspension was prepared by adding 200 μL binding buffer in a concentration of 1×10^6/mL. In cell suspension, 10 μL AnnexinV-fluorescein isothiocyanate (FITC) was added and mixed slightly followed by 15-min incubation at room temperature in the dark, in which 5 μL propidium iodide (PI) was added for staining. Within 1 hr, flow cytometer was used for detection, and the results were obtained and analyzed using Cellquest professional software. The experiments of each group were repeated 3 times.

Western blotting

Cells collected from each group were washed twice using D-Hank's solution, which was then absorbed using bibulous paper. In each group, 150 μL pre-cooled lysis buffer was added for lysis on ice for 30 min, and cell scraper was used for collecting cell proteins in each group that were placed into the Eppendorf (EP) tubes for centrifugation at 4°C and 12000 rpm. The supernatant was drawn and transferred into new EP tubes for detecting the protein concentration via bicinchoninic acid (BCA) method. Then, 5×loading buffer was added into the EP tube and mixed, and proteins were heated at 100°C for 6 min. Thereafter, 30 μL protein was added into the isolation gel and concentration gel that had already been prepared, and electrophoresis was performed in the buffer under the appropriate voltage. After electrophoresis, the gel was closely attached to the polyvinylidene fluoride (PVDF membrane), and was transferred onto the membrane at 0°C transbuffer and 100 V for 60 min. PVDF membrane was blocked using 5% skimmed milk at room temperature for 1 hr and then was cut according to the molecular weight and incubated using the primary antibodies at 4°C refrigerator overnight. In the next day, PVDF membrane was rinsed in Tween 20+Tris buffered saline (TBST), and secondary antibody (IgG, 1:5000) was added onto the membrane for 1 hr of incubation at room temperature. After incubation, the membrane was rinsed using TBST followed by color development in Tannon 5200 immunofluorescence development system, and thereafter, gray value was determined.

Statistics

Experimental data were presented as mean ± standard deviation, and statistical analysis was performed by SPSS 16.0 (Statistical Package for Social Science). One-way analysis of variance (ANOVA) was performed for intergroup comparison, and *t*-test for comparison between two groups. P<0.05 suggested that the difference was statistically significant.

Results

Silibinin inhibited the proliferation of BGC-823 cells

MTT assay was performed for figuring out the effect of silibinin on cell proliferation in different concentrations and action duration. The results showed that, compared with the control group, silibinin could obviously inhibit cell proliferation. Comparison of inhibition rate on cell proliferation between the 25 μM group and the control group showed no statistically significant difference, but compared with the control group, other groups with different concentrations showed significant inhibitory effects on cell proliferation, and the inhibition rate was increased against the augmentation in concentration. After 24 hrs of culture, the inhibition rates of silibinin in different concentrations (25 μM, 50 μM, 100 μM, 150 μM, 200 μM) were respectively (1.56±0.78)%, (6.22±1.3)%, (13.46±1.08)%, (38.32±2.01)% and (48.12±2.5)%. Compared with those that were treated for 24 hrs, inhibition of the proliferation of cells that were treated for 48 hrs was increased, but there was no statistically significant difference. This result indicated that silibinin inhibited the proliferation of BGC-823 cells in a concentration- and time-dependent manner (Figure 1).

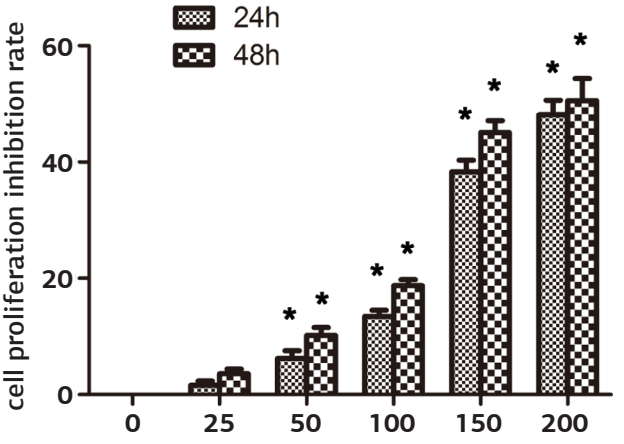

Figure 1. Detection of inhibition rate on cell proliferation via MTT assay. The final concentrations of silibinin are sequentially 0μM, 25μM, 50μM, 100μM, 150μM and 200μM. The 0μM group is set as the control of high-sugar DMEM. Detections for inhibition rate on cell proliferation were respectively carried out after 24 hours and 48 hours of culture.*Compared with the control group, p<0.05.

Silibinin enhanced the cytotoxic effect on BGC-823 cells

The cytotoxic effect of silibinin on BGC-823 cells was detected via LDH assay, and the results showed that silibinin could significantly augment the release of LDH in cells. In the 25 μM group, the content of LDH in the supernatant was in-

creased compared with the control group but with no statistically significant difference, and in other groups, the release of LDH was significantly higher than that in the control group. After 24 hrs of culture in silibinin in different concentrations (25 μM, 50 μM, 100 μM, 150 μM and 200 μM), the ratios of concentrations of released LDH to that in the control group were respectively 1.22±0.78, 2.04±0.3, 2.99±0.38, 3.54±0.42 and 4.7±0.33. Compared with those groups that received treatment for 24 hrs, the release of LDH in the groups that received 48-hr treatment was increased but without any statistically significant difference, indicating that the cytotoxic effect of silibinin on cells is in a concentration- and time-dependent manner (Figure 2).

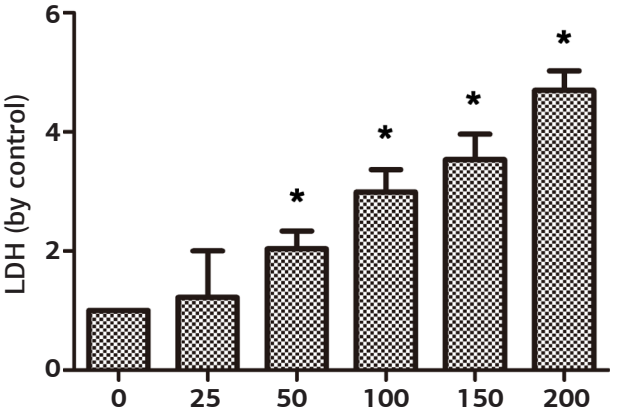

Figure 2. Detection of cytotoxic effect via LDH assay.The final concentrations of silibinin are sequentially 0μM, 25μM, 50μM, 100μM, 150μM and 200μM. The 0μM group is set as the control of high-sugar DMEM. After 24 hours of treatment using silibinin, the concentration of LDH in supernatant was detected, and with the LDH concentration measured in the control group, we calculated the ratio of LDH concentrations.*Compared with the control group, p<0.05.

Silibinin induced apoptosis of BGC-823 cells

Cell apoptosis was detected using flow cytometry. After cells were treated using silibinin in concentrations of 0 μM, 50 μM, 100 μM and 200 μM, we performed flow cytometry to detect the cell apoptosis. In the 0 μM group, the apoptotic rates in early and late stages were 1.14% and 0.58%, respectively, and the total apoptotic rate was 1.72% ; in the 50 μM group, the apoptotic rates in early and late stages were 5.4% and 1.87%, respectively, and the total apoptotic rate was 7.27% ; in the 100 μM group, the apoptotic rates in early and late stages were 15.5% and 5.37%, respectively, and the total apoptotic rate was 20.87% ; in the 200 μM group, the apoptotic rates in early and late stages were 31.49% and 9.03%, respectively, and the total apoptotic rate was 40.52% (Figure 3A). The results showed that silibinin could induce apoptosis of BGC-823 cells, and the proportions of apoptotic cells in early- and late-stage apoptosis were increased against the increment in drug concentration (Figure 3B).

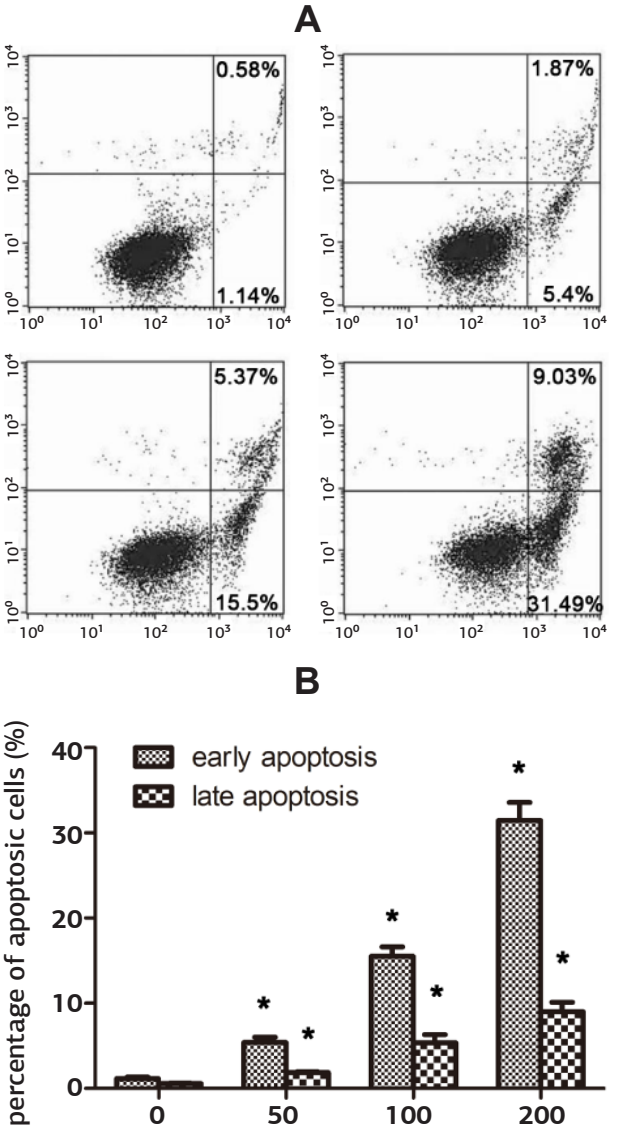

Figure 3. Detection of proportion of apoptotic cells via flow cytometry. **(A):** We detected the proportion of apoptotic cells after 24 hours of treatment using silibinin in concentrations of 0μM, 50μM, 100μM and 200μM via flow cytometry. **(B):** We performed the statistics for results of flow cytometry.*Compared with the control group, p<0.05.

Silibinin increased the expression of apoptosis-associated proteins of BGC-823 cells

Western blotting assay was used to detect the expression of apoptosis-associated proteins. Detection for apoptosis-associated proteins, Bax, Cyto C, caspase 3 and cleaved-caspase 3, was carried out after 24 hrs of treatment for cells treated with silibinin in concentrations of 0 μM, 50 μM, 100 μM and 200 μM (Figure 4A). The results showed that silibinin could significantly increase

the proportions of mitochondrial apoptosis-associated proteins Bax, Cyto C, caspase 3 and cleaved-caspase 3, and compared to the control group, the difference had statistical significance. With an increase in concentration of silibinin, the expression of apoptosis-associated proteins was increased, indicating that silibinin could accelerate cell apoptosis through inducing the initiation of mitochondrial apoptosis in a dosage-dependent manner (Figure 4B).

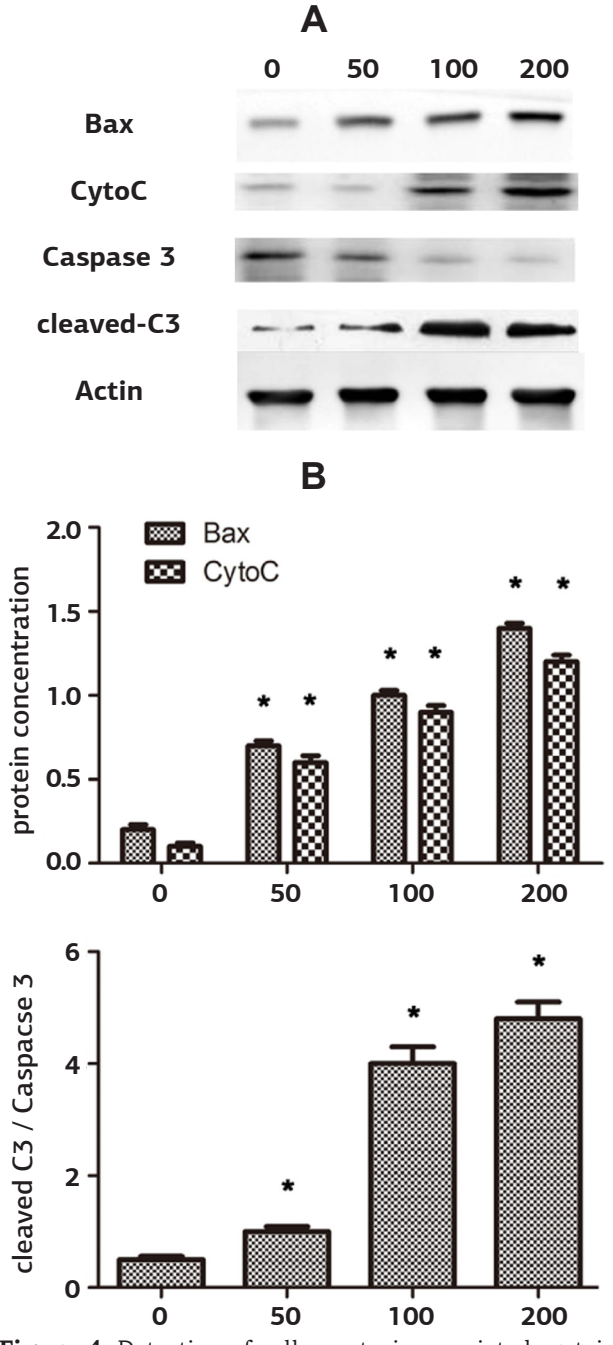

Figure 4. Detection of cell apoptosis-associated proteins via Western blotting assay. **(A):** We detected the concentrations of apoptosis-associated proteins, Bax, Cyto C, caspase-3 and cleaved-caspase 3. **(B):** We performed statistics on gray values of Bax and Cyto C proteins, and on the cleaved-caspase 3/caspase-3 ratio.*Compared with the control group, $p<0.05$.

Discussion

Although many techniques have been developed for the treatment of gastric cancer, including surgery, chemotherapy, radiotherapy, molecular targeting therapy and bio-immunotherapy, these approaches show poor therapeutic efficacy and the 5-year overall survival rate is very low. Thus, there remains a tremendous value for research on the pathogenesis and treatment methods of gastric cancer [1].

Silibinin, the flavonoids extracted from the Silybummarianum in the composite family, has a variety of physiological activities and pharmacological effects, and, in recent years, its antitumor effect has also been gradually recognized by many researchers [12]. In this study, we found that silibinin could inhibit the proliferation of BGC-823 gastric cancer cells and induce cell apoptosis; in addition, silibinin could inhibit the cell proliferation and accelerate cell apoptosis in a significant time- and dosage-dependent manner. The study by Deep et al. has shown that silibinin can inhibit the proliferation of tumor cells and induce apoptosis [13], and the relevant antitumor mechanism is associated with following factors: a) inducing differentiation of tumor cells; b) inducing apoptosis of tumor cells; c) inhibiting the phosphorylation of protein kinases (ERK1/2 and JNK1/2); d) inhibiting the expression of survivin protein and its inhibitory effect on apoptosis; e) inhibiting tumors' angiogenesis [14].

In this study, we adopted the MTT assay, and observed that silibinin could significantly inhibit the growth and proliferation of BGC-823 gastric cancer cells, and this effect was enhanced with the increase in drug concentration and extension of exposure time. The detection of LDH showed that silibinin exerts cytotoxic effect on cells, and this effect is also strengthened with the increase in drug concentration and extension of exposure time. AnnexinV. FITC/PI dual staining using flow cytometry showed that with the increase in drug concentration, the early- and late-stage apoptosis was increased, and the differences in comparison with the control group showed statistical significance, indicating that silibinin could inhibit the proliferation of BGC-823 gastric cancer cells and accelerate their apoptosis.

Caspases can be activated in a cascade-like manner through the conventional signal transduction pathway in apoptosis, finally inducing cell apoptosis, in which caspase-3 oversees the digestion of all or some of the key proteins in the final apoptotic stage; therefore, caspase-3 is also called as the final executor in apoptosis [4,15]. Howev-

er, the mitochondrial apoptosis pathway has become a hotspot in the research of cell apoptosis, and the remodeling of mitochondrial structure and function, also known as the key process in the mitochondrial apoptosis event, lead to activation of apoptosis-related proteins. When cells are stimulated by the stress factors, oligomerization will occur in the conformation of Bax protein; the oligomer of Bax protein enters the mitochondria, which can induce the formation of special channel, and facilitate the release of Cyto C from the channel into the cytoplasm. Cyto C in cytoplasm can bind to the apoptosis protease activating factor-1 (Apaf-1), which can alter the conformation of Apaf-1; further on, Apaf-1 with changes in conformation can bind to caspase-9, thereby activating the caspase-3 and initiating apoptosis [16,17]. In this study, we found that after the treatment with silibinin, the expressions of Bax and Cyto C in cells were significantly increased, and the ratio of cleaved-caspase 3 to caspase 3 was also increased, which affirmed that silibinin may induce cell apoptosis through the mitochondrial apoptosis pathway.

Conclusions

Silibinin can inhibit the proliferation of BGC-823 gastric cancer cells, and such an inhibitory effect is time- and concentration-dependent. Additionally, silibinin can promote the apoptosis of BGC-823 gastric cancer cells, which may be realized through the mitochondrial apoptosis pathway.

Conflict of interests

The authors declare no confict of interests.

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