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Original Article

Asperuloside Promotes Apoptosis of Cervical Cancer Cells through Endoplasmic Reticulum Stress-Mitochondrial Pathway*

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ABSTRACT **Objective**: To investigate the effects of asperuloside on cervical cancer based on endoplasmic reticulum (ER) stress and mitochondrial pathway. **Methods**: Different doses (12.5–800 μg/mL) of asperuloside were used to treat cervical cancer cell lines Hela and CaSki to calculate the half maximal inhibitory concentration (IC_{50}) of asperuloside. The cell proliferation was analyzed by clone formation assay. Cell apoptosis, intracellular reactive oxygen species (ROS) and mitochondrial membrane potential were determined by flow cytometry. The protein expressions of cleaved-caspase-3, Bcl-2, Bax, Cyt-c, cleaved-caspase-4 and glucose-regulated protein 78 (GRP78) were analyzed by Western blot. And the inhibitor of ER stress, 4-phenyl butyric acid (4-PBA) was used to treat cervical cancer cells to further verify the role of ER stress in the apoptosis of cervical cancer cells induced by asperuloside. **Results**: Asperuloside of 325, 650, and 1300 μg/mL significantly inhibited the proliferation and promoted apoptosis of Hela and CaSki cells ($P<0.01$). All doses of asperuloside significantly increased intracellular ROS levels, reduced mitochondrial membrane potential, significantly reduced Bcl-2 protein expression level, and increased Bax, Cyt-c, GRP78 and cleaved-caspase-4 expressions ($P<0.01$). In addition, 10 mmol/L 4-PBA treatment significantly promoted cell proliferation and reduced apoptosis ($P<0.05$), and 650 μg/mL asperuloside could reverse 4-PBA-induced increased cell proliferation, decreased apoptosis and cleaved-caspase-3, -4 and GRP78 protein expressions ($P<0.05$). **Conclusion**: Our study revealed the role of asperuloside in cervical cancer, suggesting that asperuloside promotes apoptosis of cervical cancer cells through ER stress-mitochondrial pathway.

KEYWORDS asperuloside, Chinese medicine, cervical cancer, endoplasmic reticulum stress, mitochondrial damage, apoptosis

Cervical cancer is a common malignant tumor of the female reproductive system with a high degree of malignancy and rapid progression, which seriously affects the normal life of patients.[1] According to statistics, there were about 570,000 new cases and 310,000 deaths worldwide in 2018.[2] The main cause of cervical cancer is related to human papillomavirus (HPV) infection, 70% of invasive cervical cancer is related to HPV16 and/or 18 infections,[3] and other risk factors include immunocompromised, smoking and passive smoking, oral contraceptives, etc.[4] At present, the early treatment of cervical cancer is mainly surgery, and some early patients with high-risk factors for postoperative recurrence and patients with intermediate and advanced cervical cancer all need radiotherapy and chemotherapy, however, the treatment of cervical cancer still has certain limitations.[5] Finding a method with fewer side effects and harm to the human body to treat cervical cancer is still an urgent clinical need.

Plant extracts and their derivatives have attracted more and more attention as natural medicinal products due to their low side effects.[6,7] Asperuloside (ASP) is an iridoid glycoside, mainly derived from *Rubia Tinctorum* L. (Rubiaceae), such as *Hedyotis diffusa*, which has a wide range of pharmacological effects.[8] In recent years, studies have shown that ASP has good anti-cancer activity, and has cytotoxicity to human breast cancer, leukemia and oral cancer cells, which can be used as a potential anticancer compound.[9,10]

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The studies on the anti-cancer activity of ASP indicate that it may play a role through anti-angiogenesis, cytotoxicity, immunotherapy and anti-chromosome break.[11] However, the effects and possible mechanism of ASP on cervical cancer need to be further studied.

It is widely believed that the anti-cancer effects of various compounds are based on apoptosis.[12] The endoplasmic reticulum (ER) is an important organelle responsible for protein biosynthesis and folding, cell and calcium homeostasis, etc., and is considered to be the key to regulating cell apoptosis.[13] When unfolded proteins accumulate in the ER, cells respond by initiating ER stress to protect themselves.[14,15] However, when there is long-term ER stress or correction deficiency, cells will undergo apoptosis through the mitochondrial pathway.[16] ER stress has been shown to be abnormal in many cancers, and GRP78 has been reported to be up-regulated in many cancers.[17-19] ER-mediated unfolded protein response and mitochondrial apoptosis are potential targets for cancer therapy.[20] In this study, human cervical cancer cell lines Hela and Ca Ski were used to investigate the effects of ASP on the apoptosis of cervical cancer cells.

METHODS

Cell Culture and Treatment

Human cervical cancer cell lines Hela (Cat. No. CL-0101) and Ca Ski (Cat. No. CL-0048) were purchased from Procell Life Science & Technology Co., Ltd. (China) and authenticated by STR profiling. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, USA) containing 10% fetal bovine serum (FBS, HyClone, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, USA) with an incubator at 37 ℃ with 5% CO_2.

ASP (CAS: 14259-45-1) was obtained from the resources platform of the national standard material (Cat. No. D107234, purity ⩾98%; China). Hela and Ca Ski cells were treated with 0.1% dimethyl sulfoxide (DMSO) as the control and 12.5, 25, 50, 100, 200, 400, 800 μg/mL ASP for 24 h, respectively, and the half maximal inhibitory concentration (IC_{50}) of ASP was detected. The subsequent experiment was divided into two parts. In the first part of the experiment, cells were divided into 4 groups: control, 325, 650 and 1,300 μg/mL (approximately equal to the $1/2 \times IC_{50}$, IC_{50} and $2 \times IC_{50}$) ASP groups, respectively, to analyze the effects of ASP on cervical cancer. In the second part of the experiment, cells were divided into 4 groups: control, 4-phenyl butyric acid (4-PBA, Sigma, USA, 10 mmol/L), ASP (652 μg/mL) and 4-PBA + ASP groups. 4-PBA is an ER stress inhibitor, and this part was used to confirm the role of ER stress on the apoptosis of cervical cancer cells inducted by ASP.

Cell Counting Kit-8 Detection

Hela and Ca Ski cells were inoculated into 96-well culture plates at a density of 5×10^3 per well and treated with different concentrations of ASP (0, 12.5, 25, 50, 100, 200, 400, 800 μg/mL) for 24 h. Cell viability was detected using a cell counting kit-8 (CCK-8; Beyotime, China) at 37 ℃ for 1 h. The absorbance was measured at 450 nm using a microplate spectrophotometer (cat. No. 1681150; Bio-Rad, USA) and cell viability was calculated as follows: Cell viability (%) = $(A_{experimental} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$. The concentration-effect curve was drawn according to cell viability, and the IC_{50} of ASP in Hela and Ca Ski cells was obtained.

Clone Formation Assay

Colony formation analysis was used to analyze the effect of ASP on the proliferation of cervical cancer cell lines Hela and Ca Ski. Hela and Ca Ski cells undergoing different treatments were inoculated into 6-well culture plates and incubated for 14 days under normal growth conditions to form colonies. Then the cells were fixed with 4% paraformaldehyde (Beyotime) for 15 min, stained with 0.1% crystal violet (Beyotime) and counted.

Apoptosis Analysis

Annexin V-APC/PI apoptosis kit (Elabscience, China) was used to analyze the apoptosis of cervical cancer cells under different treatments by flow cytometry. The Hela and Ca Ski cells undergoing different treatments were collected and centrifuged at 1,000 r/min for 5 min, then resuspended with phosphated buffered saline (PBS). After counting, 1×10^5 resuspended cells were centrifuged and resuspended with 500 μL 1 × Annexin V binding buffer. The Annexin V-APC and propidium iodide (PI) dyeing solution were added and stained for 15 min. Then the fluoresence was immediately examined by flow cytometry with Annexin V-APC selected APC channel and PI selected PE channels (Becton, Dickinson and Company, USA).

Intracellular Reactive Oxygen Species Detection

Reactive oxygen species (ROS) are involved

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in the regulation of cell signaling pathways including cancer cell apoptosis.[21] Hela and Ca Ski cells undergoing different treatments were collected, cell suspension concentration was adjusted to about 1×10^6/mL, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe (Cat. No. CA1410; Solarbio, China) with a final concentration of 10 pmol/L was added and incubated at 37 ℃ for 20 min. The cells were washed 3 times with a serum-free cell culture medium to fully remove DCFH-DA that did not enter the cells. Fluorescence intensity was detected by flow cytometry (Becton, Dickinson and Company).

Mitochondrial Membrane Potential Detection

Mitochondrial membrane potential assay kit with JC-1 (Cat. No. C2006, Beyotime) was used to analyze the changes of mitochondrial membrane potential in cervical cancer cells treated with different doses of ASP. About 5×10^4 cells with different treatments were taken and re-suspended in 0.5 mL cell culture medium. Then 0.5 mL JC-1 staining solution (Beyotime) was added to the cell suspension and incubated at 37 ℃ for 20 min. After incubation, the cells were centrifuged at 1,000 r/min for 5 min at 4 ℃, and the supernatant was discarded. Then cells were washed with 1 × JC-1 staining buffer for twice, then suspended with 1 × JC-1 staining buffer and analyzed by flow cytometry (Becton, Dickinson and Company).

Western Blot Analysis

In order to investigate whether ER stress is involved in ASP-induced apoptosis of cervical cancer cells, the expression of ER stress marker protein glucose-regulated protein 78 (GRP78) and ER stress-induced apoptotic protein cleaved-caspase-4 was detected by Western blot. Hela and Ca Ski cells undergoing different treatments were collected separately. Total cellular proteins were obtained by lysing cells in radio immunoprecipitation assay (RIPA) buffer (Cat. No. P0013, Beyotime) and the proteins were determined by bicinchoninic acid (BCA) protein assay kit (Cat. No. P0009, Beyotime). Protein samples were denatured, separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto poiy vinylidene fluoride (PVDF) membranes (Cat. No. ISEQ00010, Sigma-Aldrich, USA). The membranes were blocked for 1 h at room temperature in TBST containing 5% skimmed milk, then incubated overnight at 4 ℃ with anti-β-actin (1:100,000; Cat. No. AC026; Abclonal, China), anti-Cleaved-caspase-3 (1:2000; Cat. No. A2156; Abclonal), anti-Bcl-2 (1:2,000; Cat. No. A19693; Abclonal), anti-Bax (1:2,000; Cat. No. A19684; Abclonal), anti-Cyt-c (1:2,000; Cat. No. A1561; Abclonal), anti-Cleaved-caspase-4 (1:2,000; Cat. No. A19305; Abclonal), anti-GRP78 (1:10,000; Cat. No. ab108615; Abcam, USA) antibodies. After washing 3 times with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:4,000; Cat. No. ab6721; Abcam) for 2 h at room temperature. Proteins on the membranes were visualized with an enhanced chemiluminescence detection kit (Cat. No. KF001, Affinity, China) using Tanon 5200 Automatic chemiluminescence image analyzer (Tanon, China).

Statistical Analysis

All experimental data were analyzed using SPSS 20.0 software, and expressed as mean ± standard deviation ($\bar{x} \pm s$). The differences among groups were statistically compared by one-way analysis of variance (ANOVA) and Tukey HSD *post hoc*, and $P<0.05$ was considered statistically significant. All experiments were independently repeated at least 3 times.

RESULTS

Effects of ASP on Cell Proliferation and Apoptosis of Cervical Cancer Cells

To explore the effects of ASP on cervical cancer, cervical cancer cell lines Hela and Ca Ski were treated with different doses of ASP (0, 12.5, 25, 50, 100, 200, 400, 800 μg/mL), and it was found that ASP could inhibit the proliferation of both Hela and Ca Ski cells in a dose-dependent manner, with IC_{50} of 683.0 μg/mL in Hela cells and 639.8 μg/mL in Ca Ski cells (Figure 1). Then, Hela and Ca Ski cells were treated with 325, 650 and 1,300 μg/mL (approximately equal to the $1/2 \times IC_{50}$, IC_{50} and $2 \times IC_{50}$) doses of ASP respectively to analyze the effects of ASP on proliferation and apoptosis of cervical cancer. The clone formation assay results indicated that

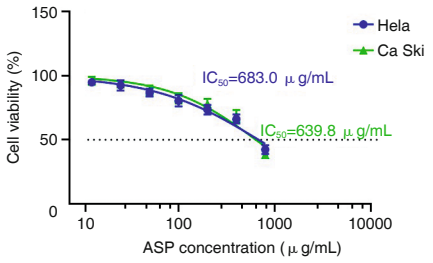

Figure 1. Concentration-Effect Curve of ASP in Hela and Ca Ski Cells

Notes: Cervical cancer cell lines Hela and Ca Ski were treated with different doses of ASP (0, 12.5, 25, 50, 100, 200, 400, 800 μg/mL). ASP: asperuloside

ASP significantly inhibited the proliferation of Hela and Ca Ski cells compared with the control group in a dose-dependent manner (Figures 2A and 2B, $P<0.01$). And the flow cytometry analysis showed that the apoptosis rates of Hela and Ca Ski cells were significantly increased in all doses of ASP compared with the control group (Figures 2C and 2D, $P<0.01$). Western blot analysis showed that the protein expression of cleaved-caspase-3 was significantly increased in Hela and Ca Ski cells after ASP treatment (Figures 2E and 2F, $P<0.01$).

ASP Promoted Cervical Cancer Cells Apoptosis through Mitochondrial Pathway

ASP could significantly increase intracellular ROS level in a concentration-dependent manner (Figure 3A, $P<0.01$). When ROS levels rise to a certain level, mitochondrial transmembrane potential decreases and nuclear apoptosis is initiated. Compared with the control group, ASP significantly reduced the mitochondrial membrane potential of cervical cancer cells (Figures 3B and 3C, $P<0.01$). In addition, ASP

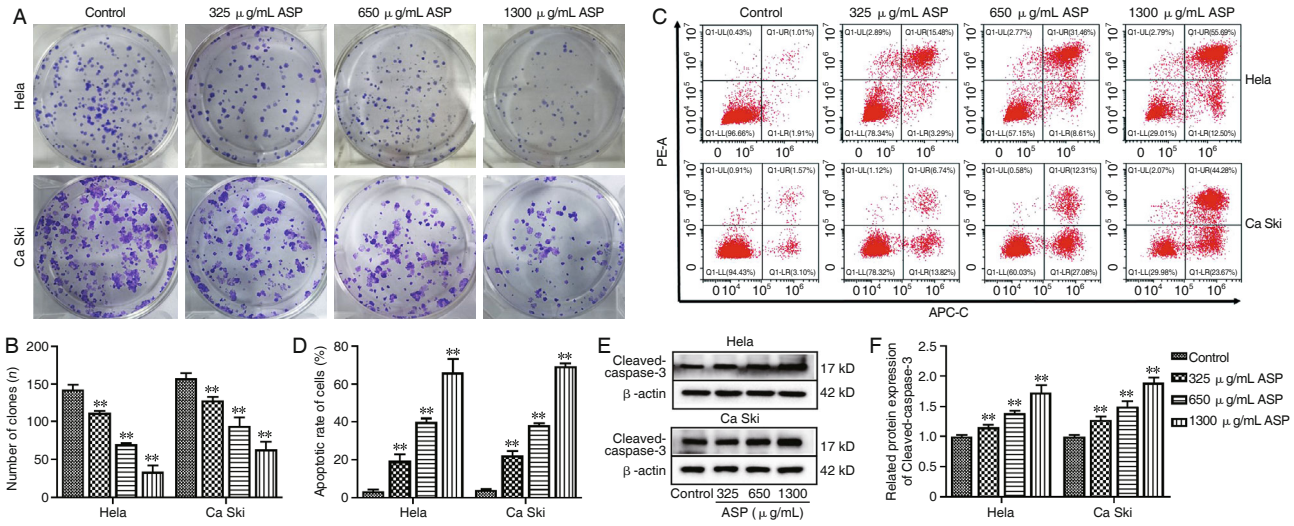

Figure 2. ASP Inhibited Cell Proliferation and Promoted Apoptosis in Cervical Cancer

Notes: (A) Cell proliferation of Hela and Ca Ski cells after ASP treatment was detected by clone formation assay. (B) The number of cloned cells in clone formation assay. (C) Cell apoptosis was detected by flow cytometry in Hela and Ca Ski cells. (D) Apoptotic rats of Hela and Ca Ski cells. (E) Protein expressions of cleaved-caspase-3 in Hela and Ca Ski cells were detected by Western blot. (F) Densitometry analysis of protein expression ($\bar{x}\pm s$, n=6). $^{*}P<0.05$, $^{**}P<0.01$ *vs*. control group. ASP: asperuloside

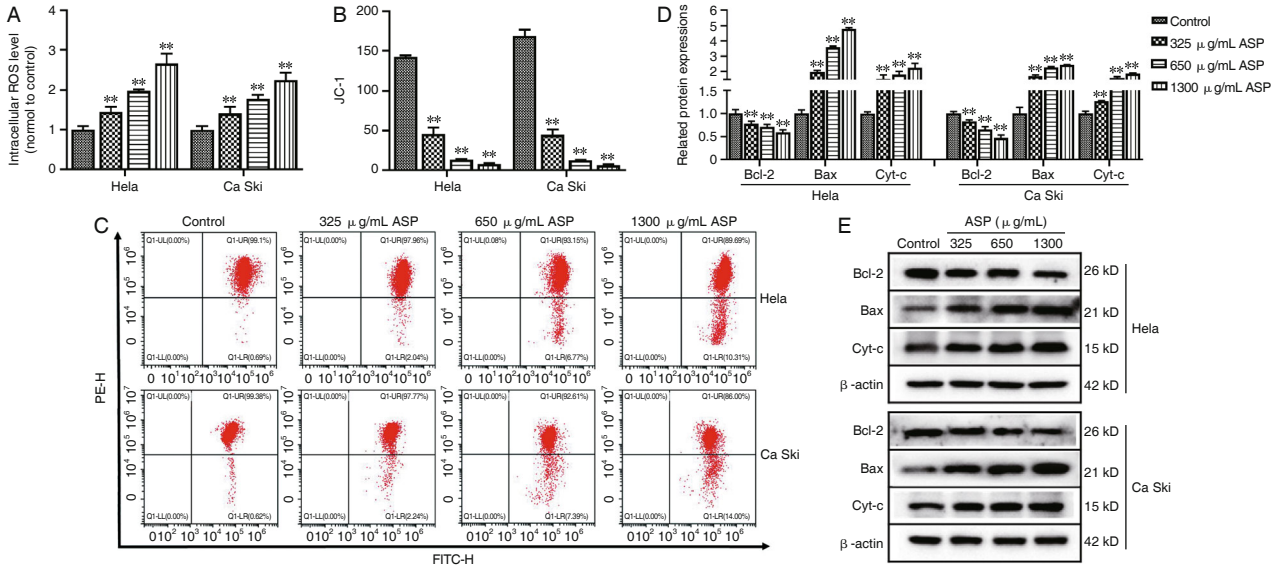

Figure 3. ASP Induced Apoptosis of Cervical Cancer Cells through Mitochondrial Pathway

Notes: (A) Intracellular ROS content was analyzed by flow cytometry using a DCFH-DA probe and homogenized with the control group. (B) The fluorescence intensity of the JC-1 polymer reflects the mitochondrial membrane potential. (C) The mitochondrial membrane potential of cervical cancer cells was determined by flow cytometry using a JC-1 probe. (D, E) Protein expressions of Bcl-2, Bax and Cyt-c in Hela and Ca Ski cells were detected by Western blot ($\bar{x}\pm$s, n=6). $^{*}P<0.05$, $^{**}P<0.01$ *vs*. control group. ASP: asperuloside

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could significantly reduce the protein expression of Bcl-2 and increase the protein expressions of Bax and Cyt-c (Figures 3D and 3E, $P<0.01$).

ASP Induced Endoplasmic Reticulum Stress in Cervical Cancer Cells

Western blot results showed that the ASP significantly increased GRP78 and cleaved-caspase-4 protein expressions in both Hela and Ca Ski cells in a concentration-dependent manner (Figure 4, $P<0.01$).

Effects of ER Stress on Apoptosis of Cervical Cancer Cells Induced by ASP

As shown in Figure 5, 4-PBA significantly promoted the proliferation and inhibited the apoptosis of Hela and Ca Ski cells ($P<0.05$). ASP could significantly reverse the increase in cell proliferation and decrease in apoptosis induced by 4-PBA, and significantly decrease the increased expression of cleaved-caspase-3, cleaved-caspase-4 and GRP78 induced by 4-PBA ($P<0.05$).

DISCUSSION

Cervical cancer is a growing problem due to high incidence, high mortality and limited availability of effective treatments.[22] Medicinal natural products

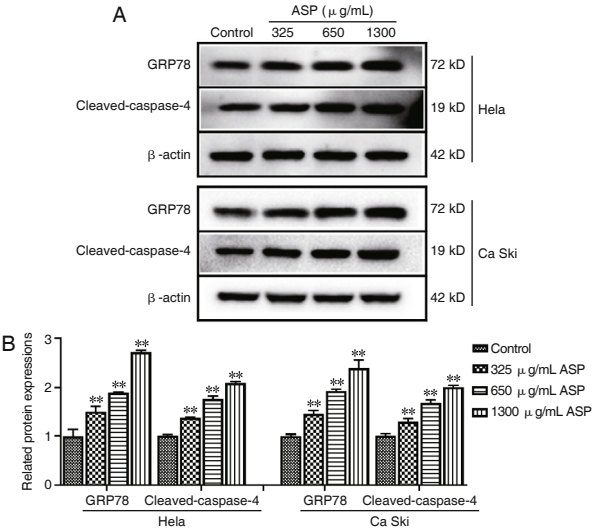

Figure 4. Effects of ASP on ER Stress of Cervical Cancer Cells by Western Blot ($\bar{x}\pm s$, n=6)

Notes: $^{*}P<0.05$, $^{**}P<0.01$ *vs*. control group. ASP: asperuloside

have been widely studied and used in the treatment of various diseases. ASP is a common secondary metabolite of iridoid glycosides and has a wide range of pharmacological effects.[11] The present study found that ASP plays an important role in cervical cancer, which can inhibit the proliferation of cervical cancer cells and promote cell apoptosis. ER stress

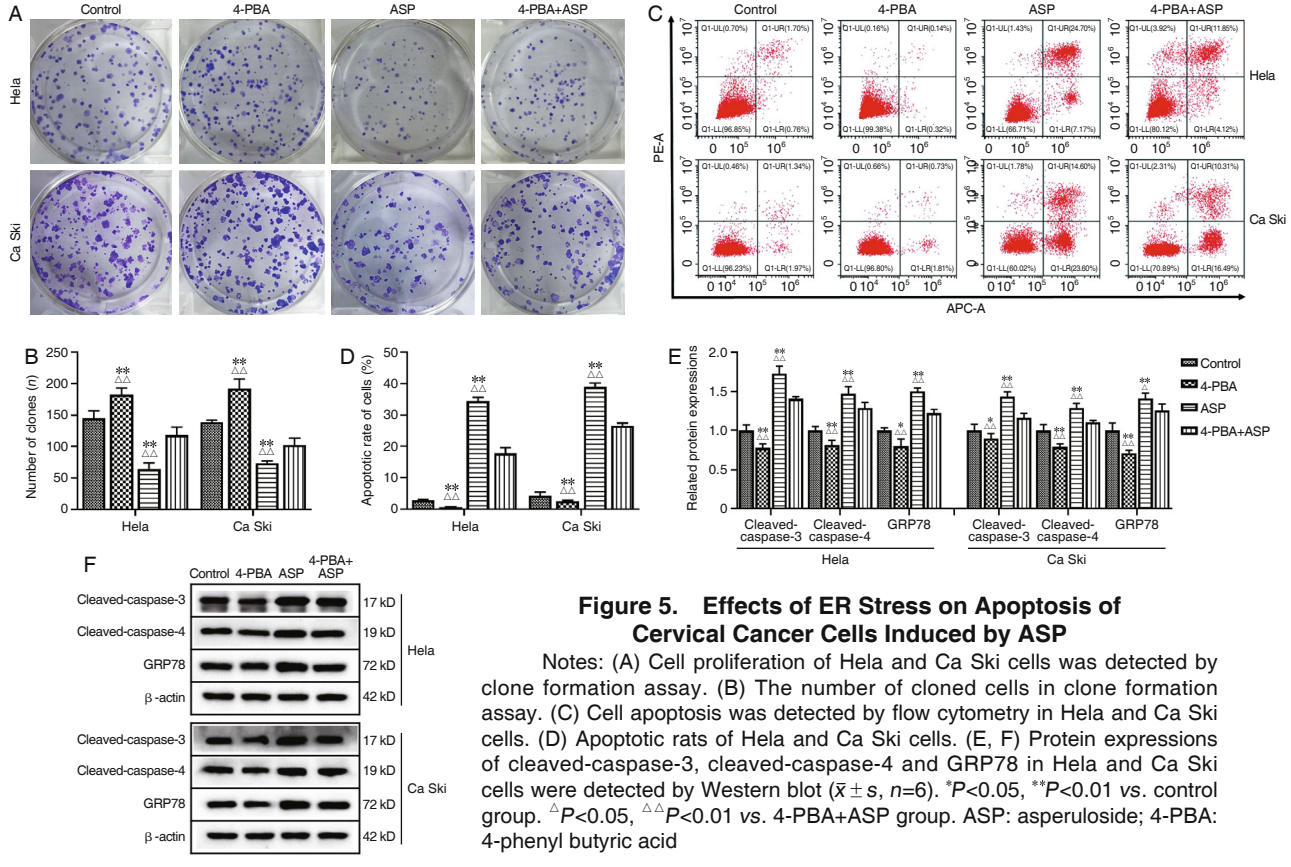

Figure 5. Effects of ER Stress on Apoptosis of Cervical Cancer Cells Induced by ASP

Notes: (A) Cell proliferation of Hela and Ca Ski cells was detected by clone formation assay. (B) The number of cloned cells in clone formation assay. (C) Cell apoptosis was detected by flow cytometry in Hela and Ca Ski cells. (D) Apoptotic rats of Hela and Ca Ski cells. (E, F) Protein expressions of cleaved-caspase-3, cleaved-caspase-4 and GRP78 in Hela and Ca Ski cells were detected by Western blot ($\bar{x}\pm s$, n=6). $^{*}P<0.05$, $^{**}P<0.01$ *vs*. control group. $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. 4-PBA+ASP group. ASP: asperuloside; 4-PBA: 4-phenyl butyric acid

and mitochondrial pathways are considered to be important pathways of cancer cell apoptosis.[23] In this study, it was found that ASP can significantly increase intracellular ROS levels, reduce mitochondrial membrane potential, and promote cell apoptosis in the mitochondrial pathway. In addition, ASP can also induce ER stress and apoptosis of cervical cancer cells. ER stress-mitochondrial pathways play important roles in the apoptosis of cervical cancer cells induced by ASP.

ASP was the first iridoid glycoside isolated from the roots of *Rubia Tinctorum* L. (Rubiaceae), and a large number of it has been demonstrated the multifaceted properties of ASP in preclinical settings.[8] In recent years, it has been shown that ASP has good anti-cancer effects.[24] ASP can destroy the formation of new cancer cells by inhibiting cell metastasis and microvascular formation,[25] and restore mammary tumor-induced osteolysis through immune regulation, resulting in a good therapeutic effect for breast cancer.[26] In addition, ASP inhibits the growth of liver cancer by regulating the release of inflammatory factors to enhance the function of immune effector cells,[27] and inhibits the growth and formation of colorectal cancer by enhancing the cytokine-induced killer cell effect, inducing apoptosis and promoting the death of apoptotic cells[28] In this study, it was found that ASP significantly inhibited proliferation and promoted apoptosis of cervical cancer cells Hela and Ca Ski, and significantly increased the expression of apoptosis protein cleaved-caspase-3 in cells, suggesting that ASP played an important role in promoting cervical cancer cells apoptosis.

Mitochondrial pathway-caused apoptosis is a classical form of cell death, and mitochondrial depolarization is a landmark event of early apoptosis.[29] Intracellular oxidative stress can trigger the decline of mitochondrial membrane potential, thereby activating caspase and initiating apoptosis.[30] In this study, it was found that ASP significantly increased intracellular ROS levels and decreased mitochondrial membrane potential, suggesting that ASP may play an important role in mitochondrial apoptosis. Apoptosis of the mitochondrial pathway is mainly regulated by Bcl-2 family proteins.[31] Changes in mitochondrial membrane permeability lead to up-regulated expression of pro-apoptotic protein Bax, while down-regulated expression of anti-apoptotic protein Bcl-2 in the Bcl-2 family, and Cyt-c and Bax are released into the cytosol to promote apoptosis.[32] The results of this study showed that the protein expression of Bcl-2 was significantly decreased, and the protein expressions of Bax and Cyt-c were significantly increased in cervical cancer cells after ASP treatment, which confirmed that ASP could promote the mitochondrial pathway apoptosis.

ER is a dynamic organelle that regulates many cellular functions by interacting with mitochondria.[16] Studies have confirmed that ER stress is also closely related to the apoptosis of cancer cells.[33] GRP78, also known as BiP, is a member of the heat shock protein 70 family and acts as a molecular chaperone. An elevated GRP78 level is a key signal of ER stress.[34] Caspase-4 is mainly located in the outer membrane of ER, and has been confirmed to play a key role in ER stress-induced human cell apoptosis.[35] The present study found that the protein levels of GRP78 and cleaved-caspase-4 in cervical cancer cell lines Hela and Ca Ski were significantly increased after treatment with ASP, suggesting that ASP promoted ER stress-induced apoptosis. In addition, 4-PBA acts as an inhibitor of ER stress and primarily acts as a chemical chaperone, protecting proteins from aggregation, promoting protein folding, and reducing ER stress.[36,37] The present study found that ASP could significantly reverse the inhibitory effect of 4-PBA on the apoptosis of cervical cancer cells, further confirming that ER stress plays an important role in the ASP-induced apoptosis of cervical cancer cells.

In conclusion, ASP significantly inhibited the proliferation of cervical cancer cells and promote apoptosis in a dose-dependent manner. ASP also increased intracellular ROS levels, reduced mitochondrial membrane potential, and significantly induced mitochondrial damage and ER stress. In addition, ER inhibitor 4-PBA could induce cervical cancer cell proliferation and inhibit apoptosis, and the inhibiting effect of 4-PBA on cervical cancer cell apoptosis can be reversed by ASP. These results suggest that ASP can inhibit the proliferation and promote apoptosis of cervical cancer cells, and the pro-apoptotic effect may be exerted through ER stress-mitochondrial pathway. However, this study was only conducted *in vitro*, and the lack of *in vivo* experiments may be a limitation of this study. Follow-up studies should use animal models to

carry out *in vivo* experiments to further verify the anti-cervical cancer effects of ASP, so as to lay a foundation for the clinical application of ASP.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Qi ZM and Zhao J conceived and designed the research. Qi ZM and Wang X performed most of the experiments. Liu X performed parts of the experiments and analyzed the data. Qi ZM and Zhao J wrote the manuscript. All authors agree to be held accountable for all aspects of the research and confirm that the data are accurate. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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