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Original Article

Paeonol induces apoptosis of ovarian cancer cells through the AKT/GSK-3β signaling pathway

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Abstract: Cisplatin-centered systemic chemotherapy is generally considered the essential method of ovarian cancer therapy. However, due to the high proportion of chemotherapy resistance and the associated side effects of chemotherapy, some patients choose to quit the systemic chemotherapy rather than to suffer through the therapeutic process. The overall survival (OS) remains dispiritedly unchanged for the past few decades. Paeonol is a main effective element extracted from a Traditional Chinese herbal medicine, the root cortex of Paeonia suffruticosa Andrews, and it has many pharmacological effects. In our research, we investigated the underlying mechanisms of Paeonol in suppressing proliferation and inducing apoptosis. We also aimed to ascertain whether its antineoplastic effect is associated with the activation of caspase-3/9 and the AKT/GSK-3β signaling pathway in ovarian cancer cells. We observed anticancer effects with proliferation inhibition and induced apoptosis on A2780 cells in a dose- and time-dependent manner after treatment with Paeonol. These effects were associated with decreased levels of phosphorylated-Akt and phosphorylated-GSK-3β. Flow cytometry results showed that the 'S' phase fraction increased while 'G0/G1' and 'G2/M' fractions decreased in a dose-dependent manner compared to the control after treatment with Paeonol for 48 h. In addition, Western blot analysis showed that treatment with increasing doses of Paeonol led to up-regulation of cleaved caspase-3/9 in protein expression, suggesting that apoptotic proteins caspase-9 and -3 directly mediate Paeonol induced ovarian cancer cells apoptosis, and their activations are essential. In conclusion, our study supports the prescription of Paeonol as a nutritional supplement for synergistic antineoplastic effects against ovarian cancer.

Keywords: Epithelial ovarian cancer, paeonol, A2780 cells, apoptosis, caspase-3/9, AKT/GSK-3β signaling pathway

Introduction

Epithelial ovarian cancer is the leading lethal gynecologic malignance and is the fifth most common cause of cancer mortality in women in the United States. In 2016, it is estimated that there are more than 22,280 new diagnoses and 14,240 deaths from this tumor in the United States [1]. According to the NCCN (National Comprehensive Cancer Network) Guidelines of Ovarian Cancer (Version 1.2016), Primary treatments for ovarian cancer are appropriate surgical staging and cytoreduction followed by systemic chemotherapy. There has been some improvement in the overall survival (OS) since the application of platinum and Paclitaxel therapy. However, the contribution to new therapies for treating women with advanced, persistent, or recurrent ovarian adenocarcinoma has remained dispiritedly stagnant for four decades [2]. In addition, because of the adverse reactions to chemotherapeutic agents, same patients choose not to suffer the misery and quit systemic chemotherapy. The 5-year survival rate is less than 30% [3]. Therefore, it is evidently necessary to screen new compounds with higher efficiency and lower toxicity, for the better adjuvant management of ovarian cancers.

Paeonol (2'-hydroxy-4'-methoxyacetophenone), a non-toxic flavonoid derivative, has been regarded as the main active component extracted from the Chinese herbal medicines of Moutan Cortex and Pycnostelmapaniculatum [4]. A body of accumulated evidence [5-8] has

demonstrated that Paeonol possesses many remarkable pharmacological and physiological effects, such as hypnosis, anti-oxidation, anti-inflammation, immunoregulation, sedation, antipyresis, and analgesic. Recently, an increasing number of studies [9-11] showed that Paeonol exerts an anti-neoplastic effect both in vitro and in vivo. Its effects on inhibiting the proliferation of ovarian cancer cells, however, remain unknown. In this paper, we report on a study that was designed to investigate the mechanism of the apoptosis-inducing effect of Paeonol in ovarian cancer cells, and to determine the effect Paeonol has on the protein kinase B (Akt)/glycogen synthase kinase (GSK)-3β signaling pathway.

Materials and methods

Cell lines and culture

Human epithelial ovarian cancer cell line (A2780) was purchased from China Center for Type Culture Collection.Cells were cultured in RPMI-1640 complete medium (Jenom, Hangzhou, China) with 10% fetal bovine serum (FBS; Gibco-BRL, Invitrogen Life Technologies) and 100 U/ml penicillin/streptomycin (Beyotime Institute of Biotechnology, Haimen, China), at 37°C in a humidified atmosphere containing 5% CO_2.

Cell growth and viability assay

Cells were cultured overnight in 96-well plates at a density of 2×10^3 cells/well. Then cells were treated with various concentrations of Paeonol, which was purchased from Natura Pharmaceutical Co., Ltd. (cat. no. 20090404; purity >99.0%; Ningbo, China). After 24 h, 48 h or 72 h of drug exposure, each sample was loaded in triplicate. MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 2 h. 180 μL of DMSO was added to each well to dissolve the formazine and absorbance was detected ata wavelength of 570 nm using a microplate spectrophotometer (Victor3 1420 Multilable Counter; PerkinElmer, Inc., Waltham, MA, USA). The percentage of cytotoxicity was calculated as follows: Cytotoxicity (%)=(1-OD570 of experimental well)/OD570 of control well. Paeonol inhibitory concentration (IC50) was expressed as the drug concentration at which cell growth was inhibited by 50%.

Cell cycle analysis

A2780 cells were plated at a seeding density of 3×10^5 cells/well and allowed to incubate overnight. The cells were treated with different concentrations of Paeonol. Untreated cells served as negative control. After 24 h of incubation, the cells were trypsinized and washed with phosphate buffered saline (PBS) containing no magnesium and calcium. Cells were resuspended in 400 μL staining solution containing 0.5 μg/ml RNaseA and 50 μg/ml propidium iodide (PI; Sigma, Louis, MO). Following 15 min of incubation in the dark at room temperature, the cells were analyzed using the FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed by BD FACS DIVA software (BD Biosciences).

Annexin V/PI assay

A2780 cells were cultured overnight at a seeding density of 3×10^5 cells/well and treated with 0.2, 0.4, 0.8 and 1.6 mM Paeonol for 48 h. Annexin V/PI assay was carried out according to the manufacturer's instructions (Beyotime Institute of Biotechnology). The cells were resuspended at a density of 2×10^6/ml and incubated with 10 μl Annexin V-FITC at room temperature briefly for 15 min in the dark. Subsequently, 5 μl PI was added for 5 min, following which 400 μl 1X binding buffer was added into the sample and analyzed within 30 min using flow cytometer. The percentages of early apoptotic, late apoptotic and necrotic cells were analyzed using BD FACS DIVA software.

Western blot analysis

Total protein was extracted from the A2780 cells using radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology) and the quantity was determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). The protein samples (20 μg) were separated by electrophoresis on SDS-PAGE gels (10%; Beyotime Institute of Biotechnology) and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Following blocking, the membranes were incubated overnight at 4°C with diluted primary antibody. The PVDF membranes were then washed three times with Tris-buffered saline with 0.1% Tween 20

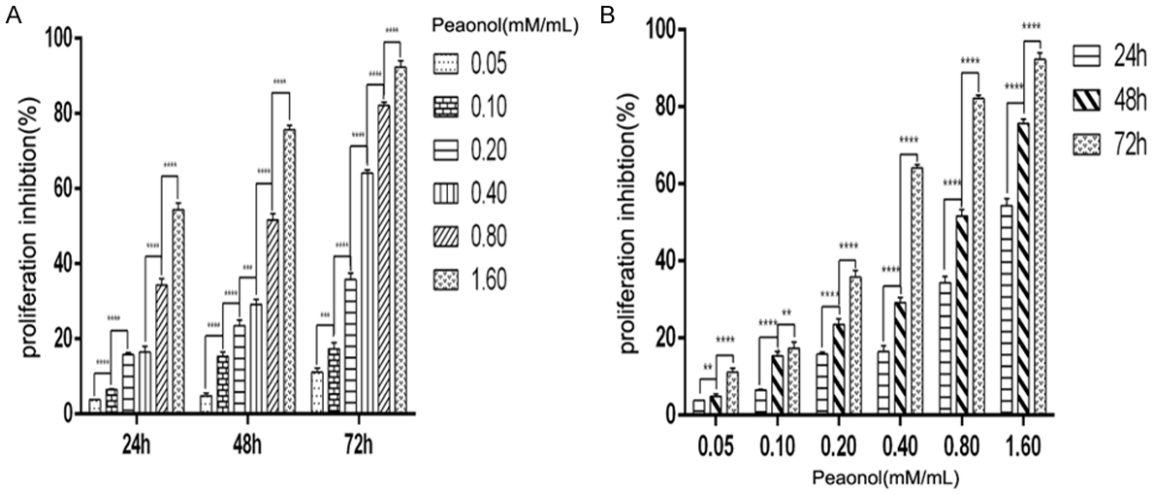

Figure 1. Peaonol induces A2780 cells proliferation inhibition. A. A2780 cells proliferation inhibition following treatment with a wide concentration range of Peaonol (0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mM) assessed by MTT assay. B. Paeonol induces viability inhibition of A2780 cells for 24 h, 48 h, 72 h, respectively. Data are expressed as the mean ± SD of three independent experiments (bars represent S.D.). (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

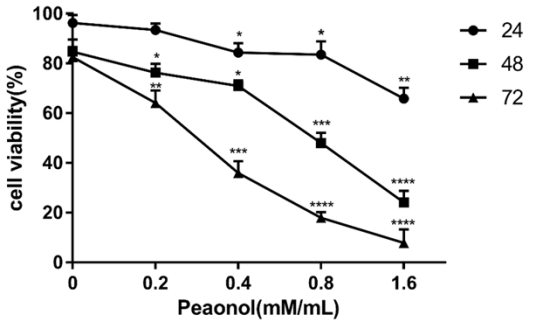

Figure 2. Peaonol induces A2780 cells viability inhibition. A2780 cells were treated with Paeonol (0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mM) for 24 h, 48 h, 72 h respectively. After the treatment, cell viability was determined by MTT assay. Data are expressed as the mean ± SD of three independent experiments (bars represent S.D.). (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, *vs* treated with 0 mM Paeonol).

(TBST; Wuhan Goodbio Technology Co., Ltd., Wuhan, China), and the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:10,000 dilution; LI-COR Biosciences, Lincoln, NE, USA) at room temperature for 1 h. Finally, the protein intensity was scanned as a fluorescent signal and quantified using an Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA). Experiments were performed in triplicate. The following primary antibodies were used: Rabbit polyclonal Akt antibody (1:500; cat. no. ab8805), rabbit monoclonal GSK-3β antibody (1:500; cat. no. ab32391), rabbit monoclonal phospho (p)-Akt (Ser473) antibody (1:500; cat. no. ab81283), rabbit monoclonal phospho (p)-GSK-3β (Ser-9) antibody (1:1000; cat. no. ab75814), all obtained from abcam, Inc. (Cambridge, USA). β-actin antibody (1:1000 dilution; cat. no. 4970; Cell Signaling Technology, Inc.) served as an endogenous reference. Images of the blots were captured using a Gel-Doc XR imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and they were analyzed using Quantity One 4.62 software (Bio-Rad Laboratories, Inc.).

Statistical analysis

The differences observed between the control and treated groups for cell proliferation, cell cycle, apoptosis and the level of the protein were analyzed using either One-way ANOVA or unpaired Student t-test (two-tailed) using the Statistical Package for Social Science (SPSS Release 22.0; SPSS Inc, Chicago, IL, USA). The results were expressed as mean ± standard deviation from triplicate experiments and a value of $P < 0.05$ was considered statistically significant.

Results

Peaonol induces proliferation and cellviability inhibition of A2780 cells

To evaluate the growth modulatory effects of Paeonol on A2780 cells, exposure to a wide

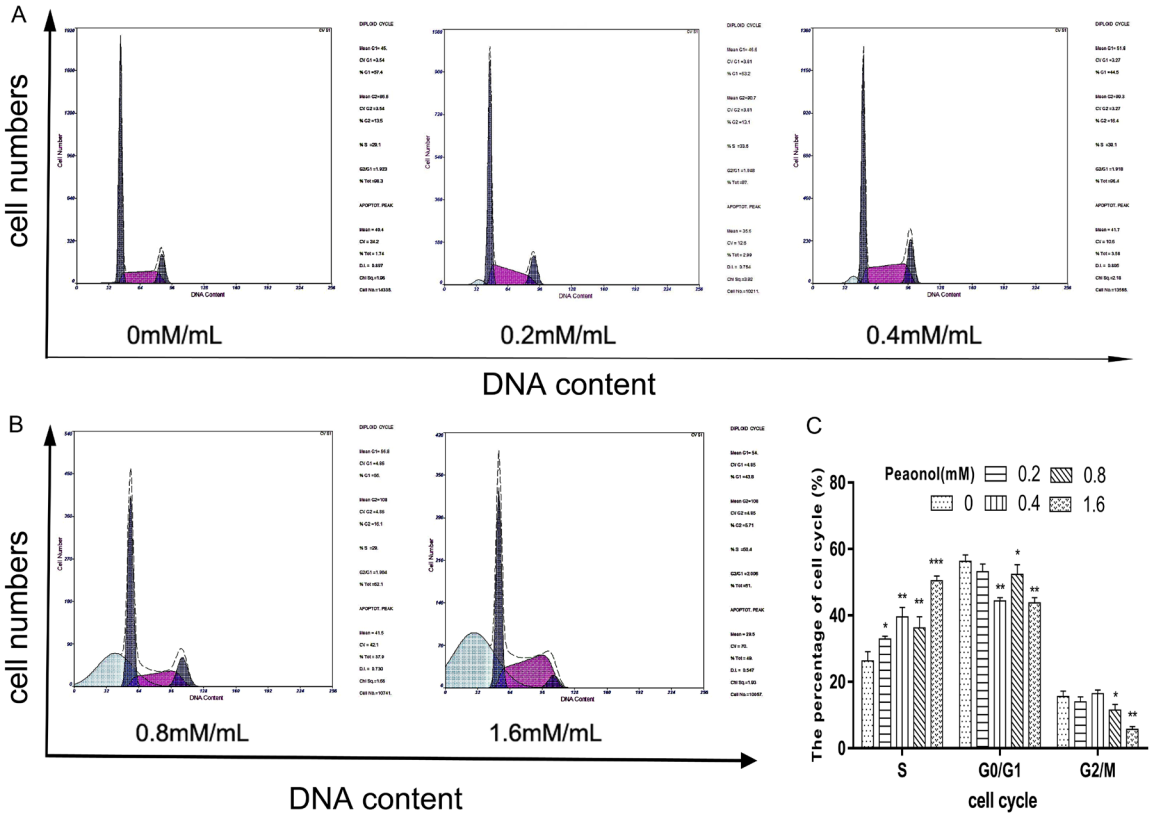

Figure 3. Peaonol induced 'S' phase arrest in A2780 cells. A and B. A2780 cells were treated for 48 h with 0.2, 0.4, 0.8, 1.6 mM Paeonol, respectively. Cells were stained with PI and analyzed for cell-cycle phase by flow cytometry after the indicated treatments. Image data is a representative of one of three independent experiments. C. The percentage of cell cycle after A2780 cells treated for 48 h with 0.2, 0.4, 0.8, 1.6 mM Paeonol. The values are expressed as mean ± SD from triplicate samples of three independent experiments. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, *vs* treated with 0 mM Paeonol).

concentration range of Paeonol (0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mM) was designed. MTT assay demonstrated A2780 proliferation inhibition following treatment with Paeonol in a dose- and time-dependent manner (**Figure 1A**, **1B**), with enhanced suppressive effects correlated to Paeonol concentration and exposure time. Modest decline in cell numbers by 3.74±0.75 percent occurred following treatment with 0.05 mM Paeonol for 24 h compared to the control. Following incubation with 1.6 mM Paeonol, there was significant suppression of cell proliferation at 24 h, 48 h and 72 h, compared with the control groups, with average rates of growth suppression of 54.28±2.04, 75.86±2.37 and 92.13±1.69%, respectively. The IC50 was detacted for the 24 h, 48 h and 72 h duration, with values of 1.44, 0.70, 0.28 mM, respectively (**Figure 2**).

Peaonol induced 'S' phase arrest in A2780 cells

The response of the cell cycle pattern of A2780 cells to treatment with various concentrations of Paeonol (0.2, 0.4, 0.8, 1.6 mM) was that the 'S' phase fraction increased while 'G0/G1' and 'G2/M' fractions decreased in a dose-dependent manner compared to the control. The percentages of cells following exposure for 48 h were 32.92%, 39.56%, 36.25%, 50.45% in 'S' phase; 55.15%, 44.38%, 52.34%, 43.75% in 'G0/G1' phase and 13.95%, 16.42%, 11.48%, 5.71% in 'G2/M' phase; for the concentrations 0.2, 0.4, 0.8, 1.6 mM Paeonol respectively (**Figure 3**).

Peaonol induced apoptosis in A2780 cells

To determine the mechanism responsible for the Paeonol-mediated suppression of cell pro-

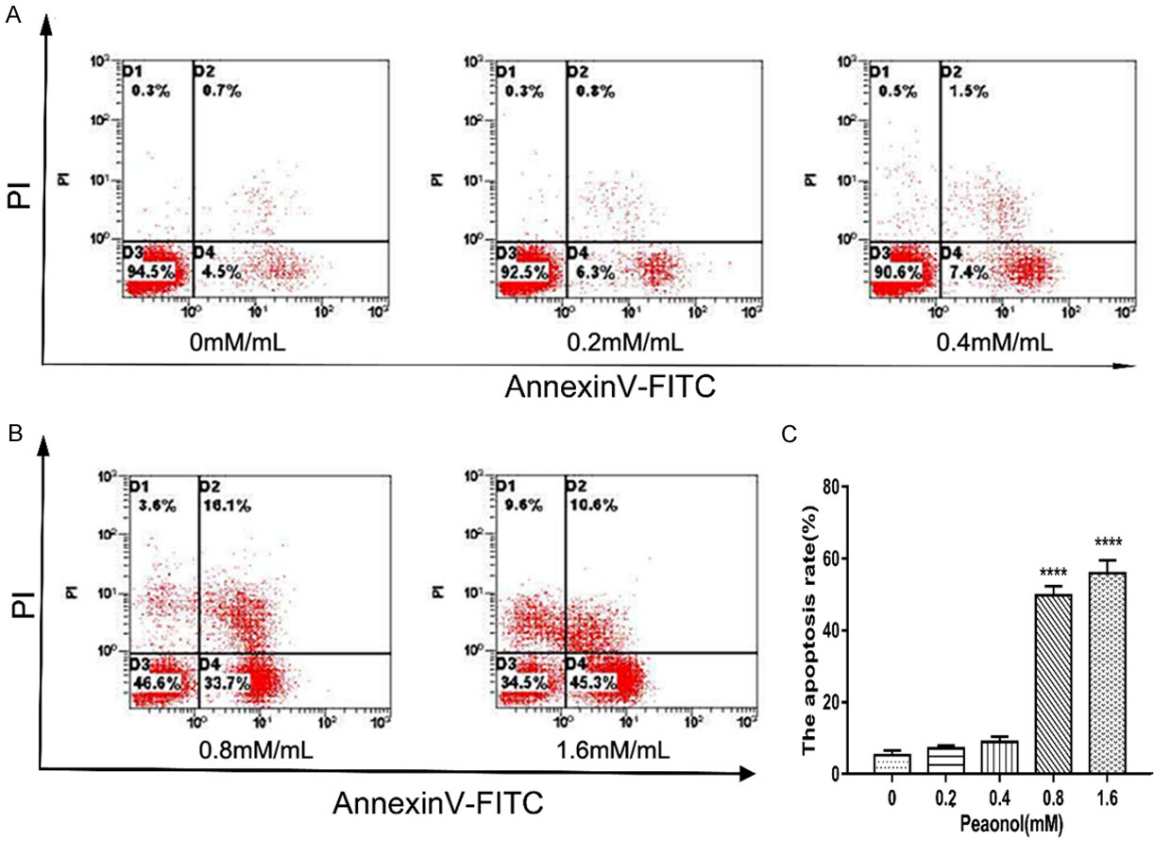

Figure 4. Peaonol induced apoptosis in A2780 cells. A2780 cells were treated for 48 h with 0.2, 0.4, 0.8, 1.6 mM Paeonol, respectively. The cells were collected sequentially and stained with propidium iodide (PI) and analyzed by flow cytometry (FCM). C. The apoptosis rate after A2780 cells treated for 48 h with 0.2, 0.4, 0.8, 1.6 mM Paeonol. The values are expressed as mean ± SD from triplicate samples of three independent experiments. (****$P < 0.0001$, *vs* treated with 0 mM Paeonol).

liferation on A2780 cells, A2780 cells treated with different concentrations of Paeonol were double-stained with Annexin V-FITC and PI. The procedure showed a dose-dependent increase in the number of apoptotic cells (**Figure 4**). The apoptosis rates were 7.14±0.81%, 8.96±1.52%, 49.85±2.46% and 55.96±3.51% for 0.2, 0.4, 0.8, 1.6 mM of Paeonol respectively, compared to control (5.24±1.38%). These increases in apoptosis were statistically significant ($P < 0.05$).

Peaonol induced the activation of Caspase-3/9 in A2780 cells

To investigate the correlation between the Caspases and the Paeonol-mediated apoptosis in ovarian cancer, A2780 cells were exposed for 48 h to Paeonol at a concentration ranging from 0.4 to 1.6 mM and expression levels of Caspase-3/9 and cleaved-Caspase-3/9 were examined by western blotting (**Figure 5A**). The expression level of caspase-3 and caspase9 showed no significant alterations, while cleaved-caspase-3 and cleaved-caspase-9, the activated form of Caspase-3 and caspase-9, showed increases in a dose-dependent manner compared to the control. The values of cleaved-caspase-3 to caspase-3 were 0.44, 0.64, 0.80 and the values of cleaved-caspase-9 to caspase-9 were 0.68, 1.06, 1.18; for the concentrations 0.4, 0.8, 1.6 mM Paeonol respectively (**Figure 5B**).

Peaonol downregulates the AKT/GSK-3β signaling pathway in A2780 Cells

To elucidate which signaling pathway is involved in Paeonol-mediated apoptosis, A2780 cells were treated with 0.4, 0.8 and 1.6 mM of Paeonol for 48 h. The results showed decreased levels of phospho-Akt and phospho-

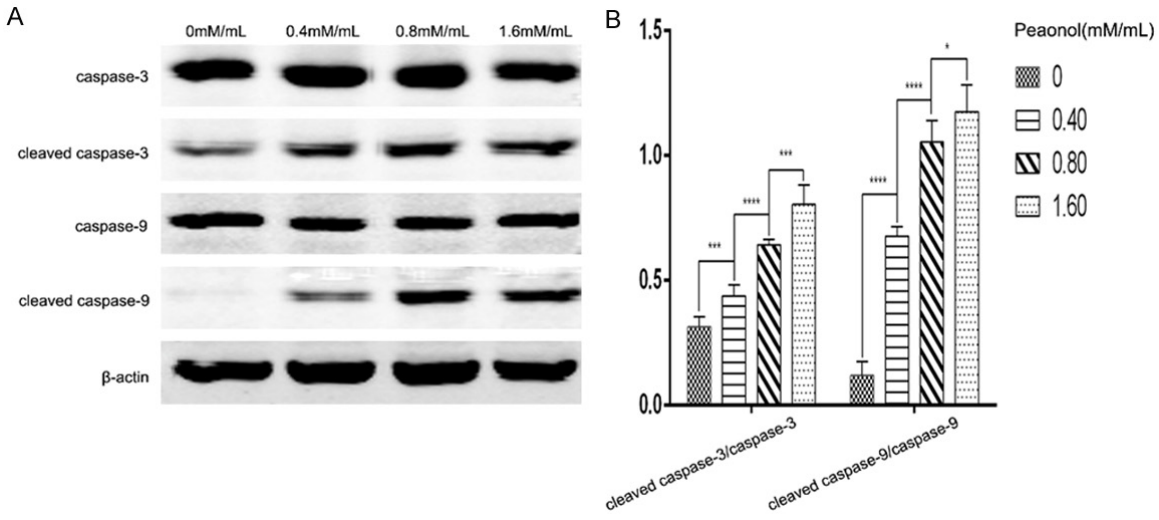

Figure 5. Peaonol induced the activation of Caspase-3/9. A. A2780 cells were exposed for 48 h with Paeonol at a concentration ranging from 0.4 to 1.6 mM and Western blot analysis showed the Caspase-3/9 and cleaved Caspase-3/9 protein expression. β-Actin was used as an internal control. B. The fold change ratio of cleaved Caspase-3/Caspase-3 and cleaved Caspase-3/Caspase-3 protein expression got increased in a dose dependent manner. The values are expressed as mean ± SD from triplicate samples of three independent experiments. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

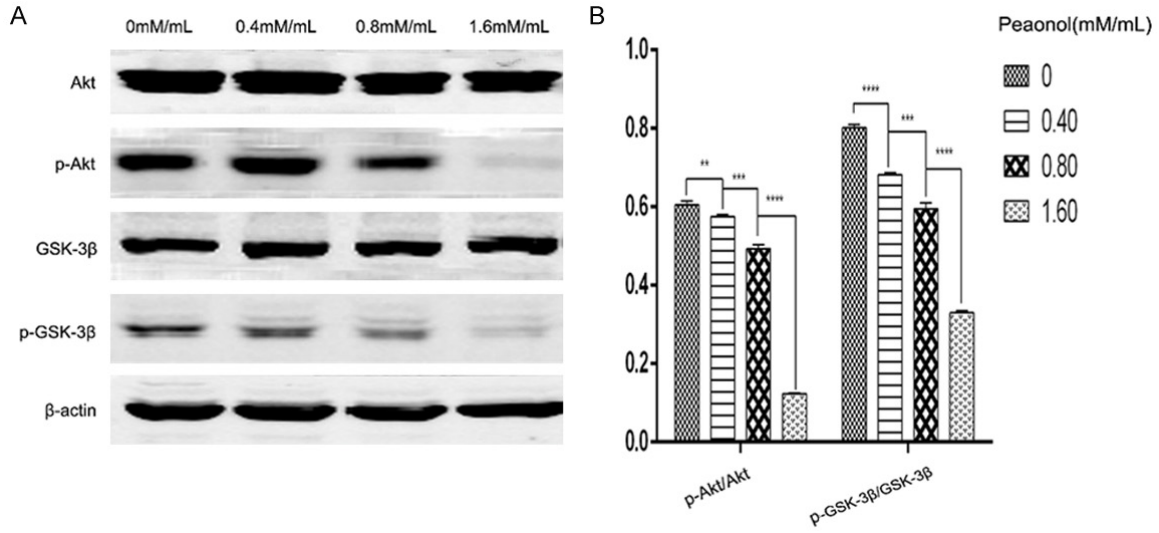

Figure 6. Peaonol downregulates the AKT/GSK-3β signaling pathway. A. A2780 cells were treated with 0.4, 0.8 and 1.6 mM of Paeonol for 48 h, and Western blot analysis showed the AKT, GSK-3β, phospho-Akt and phospho-GSK-3β protein expression. β-Actin was used as an internal control. B. The fold change ratio of phospho-Akt/AKT and phospho-GSK-3β/GSK-3β protain expression got increased in a dose dependent manner. The values are expressed as mean ± SD from triplicate samples of three independent experiments. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

GSK-3β in a dose-dependent manner, compared to the control according to the analysis of Western blotting (**Figure 6A**). In contrast, levels of total Akt and GSK-3β did not change during treatment. The fold decreases of phospho-Akt to Akt were 0.57, 0.49 and 0.12 for cells treated with 0.4, 0.8 and 1.6 mM of Paeonol respectively and these decreases were statistically significant. The fold decreases of phospho-GSK-3β to GSK-3β for 0.4, 0.8 and 1.6 mM treatment of Paeonol were 0.68, 0.58 and 0.33, respectively (**Figure 6B**).

Discussion

Cisplatin-centered systemic chemotherapy undoubtedly remains the essential method of cancer therapy. Due to the high proportion of chemotherapy resistance and the associated side effects of chemotherapy, however, cancer patients may be better advised to seek alternative forms of therapies, including natural or herbal medicines, alone or in combination with standard care. This has led to increased interest and active exploration of potential anticancer agents from Traditional Chinese medicine. Interestingly, several cancer chemotherapeutic agents were extracted from herbs, such as paclitaxel, vinblastine, aglycones, terepenoids and etoposide, Most importantly, in the last two decades some of them were applied in clinical studies for a variety of cancers including lung, liver, colon, breast and prostate cancer [12]. Paeonol, a main effective element extracted from the root cortex of Paeonia suffruticosa Andrews used in Traditional Chinese medicine, has been enthusiastically adopted by China, Japan, and other Asian countries and applied to treat various diseases including inflammation, infection, and atherosclerosis [13]. In recent years, an increasing number of studies has focused on the relationship of Paeonol and cancer. Some researches have reported that Paeonol possesses anti-neoplastic activity in various cell lines, such as human hepatoma cell line SMMC-7721, Bel-7404 and HepG2, human cervix adenocarcinoma cell line HeLa, human gastric adenocarcinoma cell line AGS and human colorectal cancer cell line HT-29 [9, 11, 14]. Further, through experiments on tumor-bearing nude mice model in vivo, Paeonol has been shown to exert significant suppression to tumor growth in a dose-dependent manner [10, 15]. In this research, we have demonstrated the anticancer effects of Paeonolon A2780 cells through inhibition to growth and proliferation. Paeonol also induced A2780 cell apoptosis in a dose- and time-dependent manner.

Inhibition of cancer cell propagation by induction of apoptosis or cell cycle arrest would be an alternative for cancer therapeutics. Cancer cells develop mechanisms to regulate signaling pathways so as to evade host immune surveillance and prevent cell death, facilitating their long-term survival [16]. The proliferative characteristic of these cells in turn result in their uncontrolled metastasis. In this study, we observed that Paeonol induced apoptosis of A2780 cells in a dosage-dependent manner, at the 0.2 and 0.4 mM level. Although Paeonol could induce the apoptosis of occurrence, the effect on A2780 is limited. At higher dose levels (0.8 and 1.6 mM), the apoptosis rate of A2780 cells in the Paeonol-exposed group was significantly higher than the control group as shown in flow cytometry analysis.

The cell cycle engine, as an integration point for information transduced through upstream signaling networks [17], plays an essential role in the uncontrolled cell proliferation that characterizes the malignant phenotype with the acquisition of genomic instability when its breaks are released and checkpoint control mechanisms are further abrogated [18, 19]. In addition to cell proliferation arrest, A2780 cell apoptosis was induced following Paeonol exposure for 48 h, as indicated by changes in the cell cycle pattern, compared with the untreated control. Significant increases in the proportion of cells in the ‘S’ phases, followed by a decrease in proportion of cells in the ‘G1/G0’ and ‘G2/M’ phase, were observed no matter whether A2780 cells were treated with Paeonol at low (0.4 mM) or high concentrations (1.6 mM). Arguably the most essential phase is ‘S’ phase, when DNA replication occurs. DNA synthesis is tightly controlled to guarantee that replication origins are not ‘fired’ more than once per cell cycle [18, 20]. As our results suggest, the mechanism of anti-proliferative effects of Paeonol on A2780 cells works through the intervention ofDNA synthesis, and blockage of the shift in the cell cycle from the ‘S’ phase to the ‘G2/M’’ phase. This mechanism is in line with most of anti-cancer chemotherapeutic drugs [18].

Caspases are cysteine proteases with well-characterized role as apoptosis executioners. Caspase-9, a highly specific protease that only cleaves a few proteins, is activated post cytochrome C release and functions to activate effector caspases. Following the caspase-9-caspase-3 cascade, caspase-3, the primary executioner of apoptotic death, is directly cleaved and activated [21-24], and it directly-cleaves numerous cellular substrates and lead to irreversibly apoptosis.Our data suggested that treatment of A2780 cells with higher con-

centration of Paeonol (0.8 and 1.6 mM) for 48 h contributed to a conspicuous up-regulation of cleaved caspase-9 in protein expression, accompanied by an increased expression of cleaved caspase-3. This implies that apoptotic protein caspase-9 and -3 directly mediate Paeonol-induced ovarian cancer cells apoptosis and their activations are essential.

The abnormal tau protein is cleaved by caspase-3 at the N-terminus, followed by a response in relevant signaling pathways. It has been well-demonstrated that PI3K/Akt/Bclantiapoptotic and survival signaling pathway plays a crucial role in apoptosis [25, 26]. Translocation to the plasma membrane and phosphorylation at the Ser-473 and Thr-308 sites are necessary processes for the activation of Akt. Once activated, Akttranslocates to a series of subcellular compartments where it phosphorylates GSK-3β, one of the important multifunctional serine/threonine kinases. And phosphorylation at the Ser-9 for Akt. In cancer cells, increased activation of GSK-3β is pro-apoptotic, whereas its suppression is anti-apoptotic [27]. Therefore, the levels of phosphorylated-AKT (Ser-473) and phosphorylated-GSK-3β (Ser-9) represent the activity of AKT and GSK-3β, respectively. In our study, A2780 cells were exposed for 48 h to Paeonol at a concentration ranging from 0.4 to 1.6 mM and levels of Akt and GSK-3β were examined by Western blotting. Treatment of A2780 cells with Paeonoldecreased the levels of phosphorylated-Akt at serine-473 and phosphorylated-GSK-3β at Ser-9 in a dose-dependent manner, especially at concentrations above 0.8 mM, while the levels of total Akt and GSK-3β did not change during treatment. The results suggest that the effect of apoptosis induced by Paeonol may occur via inhibition of the activated Akt/GSK-3β signaling pathway, which is in line with a suppression of Akt's kinase activity that results in a restriction of GSK-3β phosphorylation, which in turn activates GSK-3β [28].

On the basis of the results described above, Paeonol induces cell cycle arrest and causes cell death via apoptosis by activating apoptosis pathways in A2780 cells. Down-regulation of the expression level of phosphorylated-Akt and activation of GSK-3β appear to be responsible for the occurrence of the caspase-9-caspase-3 cascade during treatment with Paeonol. For these reasons, the antiproliferative effect of Paeonol can be mainly identified with the ability of the Akt / GSK-3β pathway to modulate apoptosis. Further, different from classical chemotherapeutic agents, Paeonol is observed to exert only mild to moderate beneficial effects against A2780 cells. In conclusion, our study provides support for prescribing Paeonol as a nutritional supplement for its synergistic antineoplastic effects against ovarian cancer.

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Disclosure of conflict of interest

None.

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