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Inhibition Effects of Paeonol on Mice Bearing EMT6 Breast Cancer through Inducing Tumor Cell Apoptosis

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***SUMMARY*. Paeonol, a phenolic component from the root bark of *Paeonia moutan*, has been identified to possess antitumor effects on mice bearing EMT6 breast cancer in our previous studies. However, the underlying mechanisms remain unknown. In the present study the molecular mechanisms of paeonol were further investigated in EMT6 mice model. The results showed that treatment of mice with 175 and 350 mg/kg/day of paeonol significantly inhibited the growth of the EMT6 tumor in mice, and induced tumor cell apoptosis which were demonstrated by light microscopy after hematoxylin and eosin staining and apoptosis analysis by flow cytometry. In addition, compared with the control group, paeonol increased the number of tumor cells in G0/G1 phase but decreased the number of cells in S and G2/M phase. Paeonol treatment (350 mg/kg body weight) also resulted in a decrease of Bcl-2 and an increase in Bax and caspase-3 expressions, which were demonstrated by immunohistochemical and western blot analysis. These results indicate that the antitumor effects of paeonol might be associated with arresting tumor cells in the G0/G1 phase, inducing cell apoptosis and regulation of the expression of Bcl-2, Bax and activation of caspase-3.**

RESUMEN. Peonol, un componente fenólico de la corteza de la raíz de *Paeonia moutan* Sims., ha demostrado poseer efectos antitumorales en ratones portadores de cáncer de mama EMT6 en nuestros estudios anteriores. Sin embargo, los mecanismos subyacentes siguen siendo desconocidos. En el presente estudio los mecanismos moleculares de peonol se investigaron en ratones modelo EMT6. Los resultados mostraron que el tratamiento de ratones con 175 y 350 mg/kg/día de paeonol inhibió significativamente el crecimiento del tumor EMT6 en ratones y se indujeron la apoptosis de células tumorales, lo que se demostró por microscopía después de tinción con hematoxilina y eosina y el análisis de la apoptosis por citometría de flujo. Además, en comparación con el grupo de control, peonol aumentó el número de células tumorales en la fase G0/G1 pero disminuyó el número de células en fase S y G2/M. El tratamiento con paeonol (350 mg/kg de peso corporal) también dio lugar a una disminución de la expresión de Bcl-2 y un aumento de Bax y caspasa-3, que se demostraron por análisis de western blot e inmunocitoquímica. Estos resultados indican que los efectos antitumorales de peonol podrían estar asociados con la detención de las células tumorales en la fase G0/G1, la inducción de la apoptosis y la regulación de la expresión de Bcl-2, Bax y la activación de la caspasa-3.

INTRODUCTION

Paeonol is a micromolecular phenolic compound and it is the main active component that has been isolated from the root bark of the plant *Paeonia moutan*, which grows in northwest and southwest area of China. Paeonol is traditionally used as a Chinese herbal medicine that has been widely used in sedation, hypnosis, antipyresis, analgesia, antioxidation, antiinflammation, anti-bacteria and to activate the blood flow and remove blood stasis [1-4]. It has been reported that paeonal has the antitumor effect and inhibits the proliferation of different tumor cell lines which include the human erythromyeloid cell line K562, the breast cancer gene cell lines T6-17, the human hepatoma cell line Bel-7404, the cervical cancer cell line Hela and the human colorectal cancer cell line HT-29. Furthermore, paeonol can enhance the anti-tumor effect against oesophageal cancer *in vitro* when it combined with cisplatin [5-8].

Previously, we reported the anti-tumor ef-

KEY WORDS: Breast cancer, Cell cycle, Effects, Pathway, Paeonol.

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fects of paeonol on mice bearing EMT6 breast carcinoma, and indicated that its mechanisms might be associated with inhibition of tumor cells in G0/G1 phase and reduction of the expression of mutant p53, Bcl-2 and C-erbB-2 protein [9], but there was no detailed report on the mechanism of paeonol against breast cancer through inducing apoptosis.

Therefore, the study was further to explore the possible mechanisms responsible for the anti-tumor activity of paeonol using EMT6 bearing mice model. The purpose was to find the therapeutic potential of paeonol for breast cancer and to provide a scientific explanation.

MATERIALS AND METHODS

Chemicals and instruments

Paeonol was obtained from Beijing Pharmaceutical University (Beijing, China). Cyclophos phamide (CTX) was purchased from HengRui pharmacy Inc. (Jiangsu province, China). Prodium Iodine RNase was obtained from sigma chemical co. (St Louis, MO, USA). Mouse anti-Bcl-2, anti-Bax and anti-caspase 3 monoclonal antibodies and Streptavidinbiotin Peroxidase immunohistochemical reagent kits were obtained from Santa Cruz Bio Inc. (Santa Cruz, CA, USA). Horseradish peroxidase-labeled rabbit antigoat IgG antibody and actin polyclonal antibody were purchased from Biosynthesis Bio. The enhanced chemiluminescence kit was purchased from Amersham Pharmacia Biotech. The EMT6 cell line was obtained from Cancer Institute of Chinese Academy of Medical Sciences. Flow cytometry was conducted using an EPICS XL model of American Beckman Coulter. All other chemicals used were of analytical reagent grade.

Animals

Fifty female Kunming mice (6 weeks-old) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences. All animal experiment was conducted in accordance with the NIH Guide for the care and Use of Laboratory Animals (NIH Publication No.80-23; revised 1978 and the number approved by Administrated-Committee of Laboratory Animals was 062310). The mice were randomLy divided into five groups. Each group had ten animals. One group was used for the preparation of EMT6 tumor cell. The other four groups were used for different treatments. Animals were housed in plastic cages with free access to food and water and maintained on a regulated environment (20 ± 2 °C).

Prepared EMT6 tumor cells

The EMT6 breast cancer cell line (10^7 cell/mL) was injected into the right fore limb in one group of mice (0.2mL/mouse). We removed solid tumor when tumor size grew to 1cm to make cell suspension and diluted to concentration of 5×10^6 cell/mL under aseptic condition in normal saline.

Animal model and treatment with drugs

Four groups of mice were all injected with 0.2 mL/mouse of 5×10^6/mL EMT6 breast cancer cells in the skin under the right fore limb. Then 24 h after tumor inoculation, one group was administered with vehicle alone (distilled water, p.o.) as the control group. One group was given cyclophosphamide (CTX, 25 mg/kg body weight, i.p.), a standard antitumor reference drug, which was designated as the positive control group (CTX group). The other two groups were administrated paeonol, low dosage (175 mg/kg body weight, p.o.) and high dosage (350 mg/kg body weight, p.o.), separately. After 15 days, all mice were weighed and killed, and then tumor was removed and weighed. According to the mean weight of tumor, the rate of tumor inhibition was calculated as follows: *The rate of inhibition (%) = (mean tumor weight of control group - mean tumor weight of treated group) /mean tumor weight of control group × 100.*

Morphology analysis of tumor tissues

Tumor specimens which collected from the control group, CTX group and paeonol group (350 mg/kg) were fixed in 10% (v/v) neutral formalin solution, dehydrated through a graded ethanol series and embedded in paraffin. Tissue sections (4 μm) were stained with hematoxyli and eosin and then examined under the light microscope.

Cell cycle and apoptosis assay by flow cytometry

The tumors which were collected from the control group, CTX group and paeonol group (350 mg/kg) were minced, single cell suspension was prepared with 200 mesh filtering, centrifuged at 956 g for 5 min, washed three times, adjusted cell concentration to 10^6 cell/mL, fixed with 70% ethanol for 30 min at 4 °C, and DNA content and cell cycle were analyzed by flow cytometry after treatment with RNase and Propidium Iodide staining for 30 min. The proportion of cells in each cell cycle and apoptosis

number were calculated. The data were analyzed with CellQuest software.

Immunohistochemical analysis for Bc1-2, Bax, and caspase-3 in tumor tissues

Tumor specimens which collected from the control group, CTX group and paeonol group (350 mg/kg) were fixed in 10% (v/v) neutral formalin solution and embedded in paraffin. Tumor sections were prepared and used to examine the expression of Bc1-2, Bax, and caspase-3 proteins. The tumor sections were stained by the standard immunohistochemical streptavidin peroxidase method which was described in the program of streptavidin peroxidasereagents kit. The cells which stained brown suggested positive cells while blue cells indicated negative cells under a microscope. Image was acquired by using Leica Application suite software (Leica Microsystems Ltd. Germany), and the average number of positive cells was counted by using Leica Qwin software (Leica Microsystems Ltd. Germany) in five randomLy selected optical fields (200 tumor cells/per field). The average positive rate was calculated as follows: *Positive rate (%) = number of positive cells/total number of cells × 100.*

Western blot analysis for Bc1-2, Bax, and caspase-3

Tumor tissues from the four groups were minced and lysed in 500 μL cell lysis buffer for 30 min, and centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was collected and protein concentrations were determined according to the Bradford method. after samples were boiled for 5 min, they were subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes by a semi-dry transfer method. The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, washed three times with TBST, and incubated with TBST containing 5% of dried skim milk and primary antibody (Bcl-2, Bax or caspase 3) for 2 h at room temperature. After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Proteins were visualized by using an enhanced chemiluminescence kit and exposed to X-ray film. At the same time, β-actin was used as an internal control for all Western blots. The intensity of protein bands was quantified by using LabWork 3.0 UVP software.

Statistical analysis

All values are expressed as mean ± S.D. One-way analysis of variance and Duncan's multiple range tests were used for determining differences among the groups, and $p < 0.05$ was regarded as statistically significant.

RESULTS

Effect of paeonol on solid tumor growth

After tumor-bearing mice were treated with two dosages of paeonol 175 or 350 mg/kg body weight and CTX for 15 days, there was no significant influence on the body weight of mice in both the paeonol group and CTX group. However, comparing with the control group, paeonol administration of 175 and 350 mg/L significantly decreased the tumor weight in a dose-dependent manner and the tumor inhibition rates reach 44.58 and 53.82%, respectively. CTX, which is the standard chemotherapeutic, produced an inhibition rate of 59.45% (Table 1).

Morphological changes of cell apoptosis

Tumor cells in the control group were arranged closely in different sizes and shapes, a little cytoblastema and bigger nucleus with a thickly staining and obvious heteromorphism,

Group	n	Treatment (mg/kg)	Body weight (g)		Mean weight of tumor (g)	Inhibition Rate (%)
			Beginning	End		
Control	10	Vehicle	19.48 ± 1.62	23.69 ± 2.31	2.49 ± 0.36	
CTX	10	25	20.12 ± 1.53	18.76 ± 1.77	1.01 ± 0.27*	59.45
Paeonol	10	175	19.68 ± 1.72	22.93 ± 1.49	1.38 ± 0.53*	44.58
	10	350	20.13 ± 1.61	23.64 ± 2.16	1.15 ± 0.24*	53.82

Table 1. The inhibitory effect of paeonol on EMT6 solid tumor (x ± s). *$p < 0.05$ as compared with control group, values are mean ± SD.

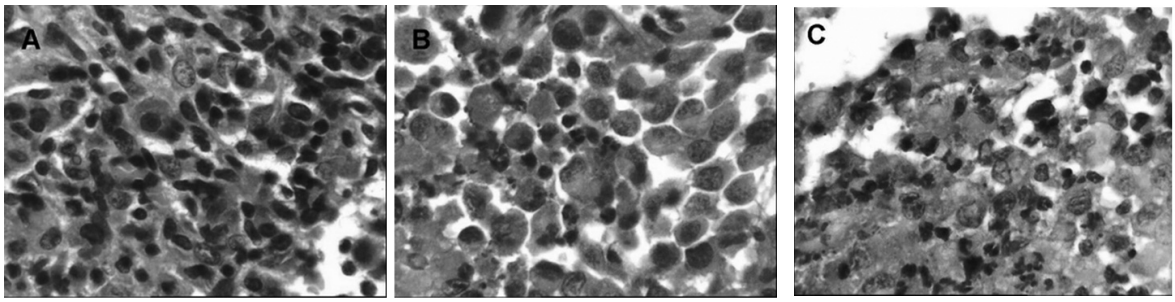

Figure 1. Morphology of EMT6 breast cacerrcinoma cell (H.E. ×400). **A**: control group; **B**: CTX group; **C**: paeonol group.

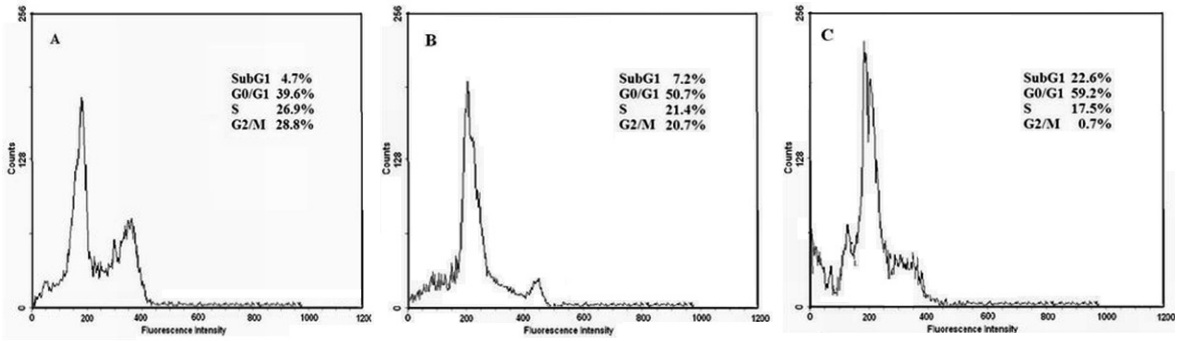

Figure 2. The effect of paeonol on cell cycle and apotosis. **A**: control group; **B**: CTX group; **C**: paeonol group.

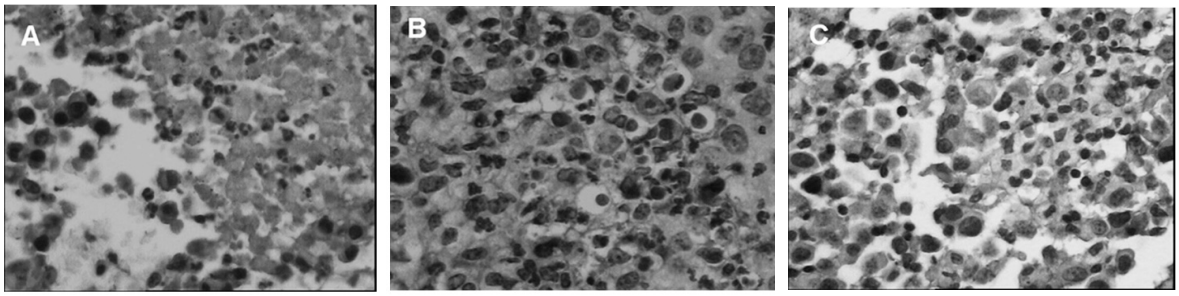

Figure 3. Effect of paeonol on the expression of Bcl-2 protein in EMT6 tumor tissues (S-P ×400). **A**: control group; **B**: CTX group; **C**: paeonol group.

and hyperplasia as well. As shown in Fig. 1, the number of tumor cells in the paeonol treatment groups decreased markedly, and tumor cell chromatin accumulated at the side of the nucleic membranes. The nucleic shape was irregular and the surface of the nucleic membrane was rough. The nucleus was broken but was encapsulated by intact membrane, containing intact organelles and apoptotic bodies. Light microscopy comparison with the control group revealed more apoptotic cells in the paeonol group.

Effect of paeonol on tumor cell cycle

After administration of CTX and paeonol (paeonol 350 mg/kg), the percentage of tumor cells in G0/G1 phase was increased significantly from 39.6% in the control group to 50.7% in CTX group and 59.2% in paeonol group. Correspondingly, the percentage of group sub-G1 cells as apoptotic cells was also significantly increased from 4.7% in the control group to 7.2% in CTX group and 22.6% in paeonol group. However, compared to the control group, CTX and paeonol reduced the proportion of tumor cells in the S and G2/M phases. These data indicated that paeonol arrested the cell cycle in G0/G1 phase and induced tumor cell apoptosis (Fig. 2).

Effects of paeonol on the expression of Bc1-2, Bax and caspase-3 proteins

The expression of Bc1-2, Bax and caspase 3

Groups	Treatment mg/kg	Bcl-2	Bax	Caspase-3
Control	Vehicle	71.24 ± 8.84	28.65 ± 6.25	33.62 ± 7.34
CTX	25	33.51 ± 7.48*	47.24 ± 7.61*	57.49 ± 8.27*
Paeonol	350	24.37 ± 5.42*	64.72 ± 9.44*	81.26 ± 6.73*

Table 2. The effect of paeonol on the expression of Bcl-2, Bax and caspase-3 (x ± s,%). *p<0.05 as compared with control group, values are mean± SD.

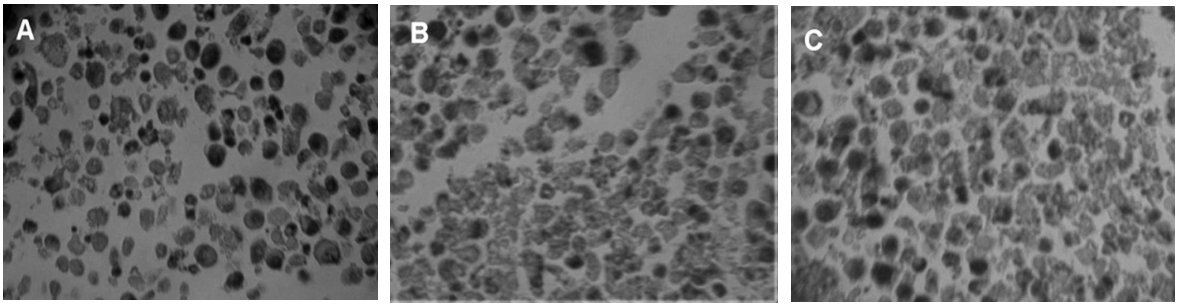

Figure 4. Effect of paeonol on the expression of Bax protein in EMT6 tumor tissues (S-P ×400). **A**: control group; **B**: CTX group; **C**: paeonol group.

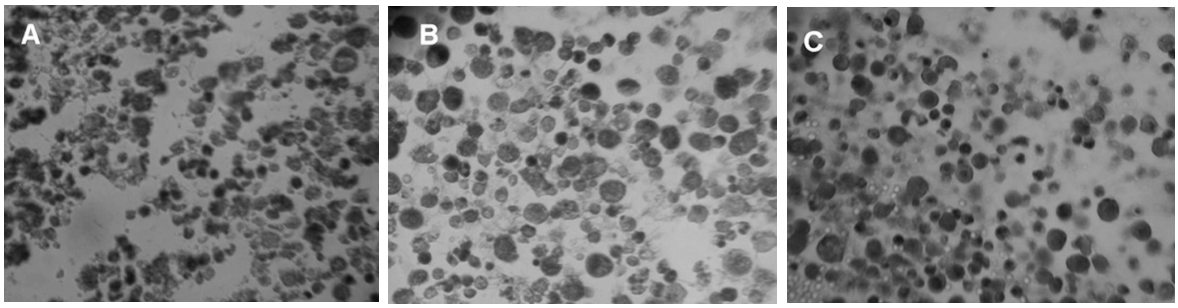

Figure 5. Effect of paeonol on the expression of Caspase-3 in EMT6 tumor tissues (S-P ×400). **A**: control group; **B**: CTX group; **C**: paeonol group.

proteins were examined with the Streptavidin Peroxidase method and Western blotting. Comparing with the control group, administered with paeonol (350 mg/kg) reduced the expression of Bc1-2 while increased the expression of Bax and caspase 3 proteins. The positive rate of Bcl-2 was 71.24% in the control group, administered with CTX and paeonol, the positive number of Bcl-2 decreased to 33.51% in CTX group and 24.37% in paeonol group (Table 2, Fig.3). The positive rate of Bax was 28.65% in the control group, after administration with CTX and paeonol, the positive number of Bcl-2 increased significantly to 47.24% and 64.72%, respectively. (Table 2, Fig. 4). Similar to Bax, the positive percentage of caspase-3 was 33.62% in the control group while the treatment of CTX and paeonol significantly increased the number of caspase-3 positive cells to 57.49% in CTX group and 81.26% in paeonol group (Table 2, Fig. 5).

The expression of Bcl-2, Bax and caspase 3 proteins were further detected using western blotting. As shown in Fig. 6, the result was similar to the immunohistochemical assay, which showed that the expression of Bcl-2 was reduced while the expression of Bax and caspase 3 was increased, and the down-regulation of Bcl-2 and up-regulation of Bax led to a decrease in the ratio of Bcl-2/Bax (Fig. 6A, B, C, D).

DISCUSSION

Breast cancer is the second most common cancer in females in the world and there are about more than 1.5 million cases and 570 thousand of patients die of breast cancer per year. Recently, the rate of incidence is the tendency of increasing [10]. The current therapeutic approaches include surgery, chemotherapy, and radiotherapy which can give patients a higher survival rate. However, the prognosis of these patients is poor because it often recurs in several years [11-13]. Therefore, to explore new com-

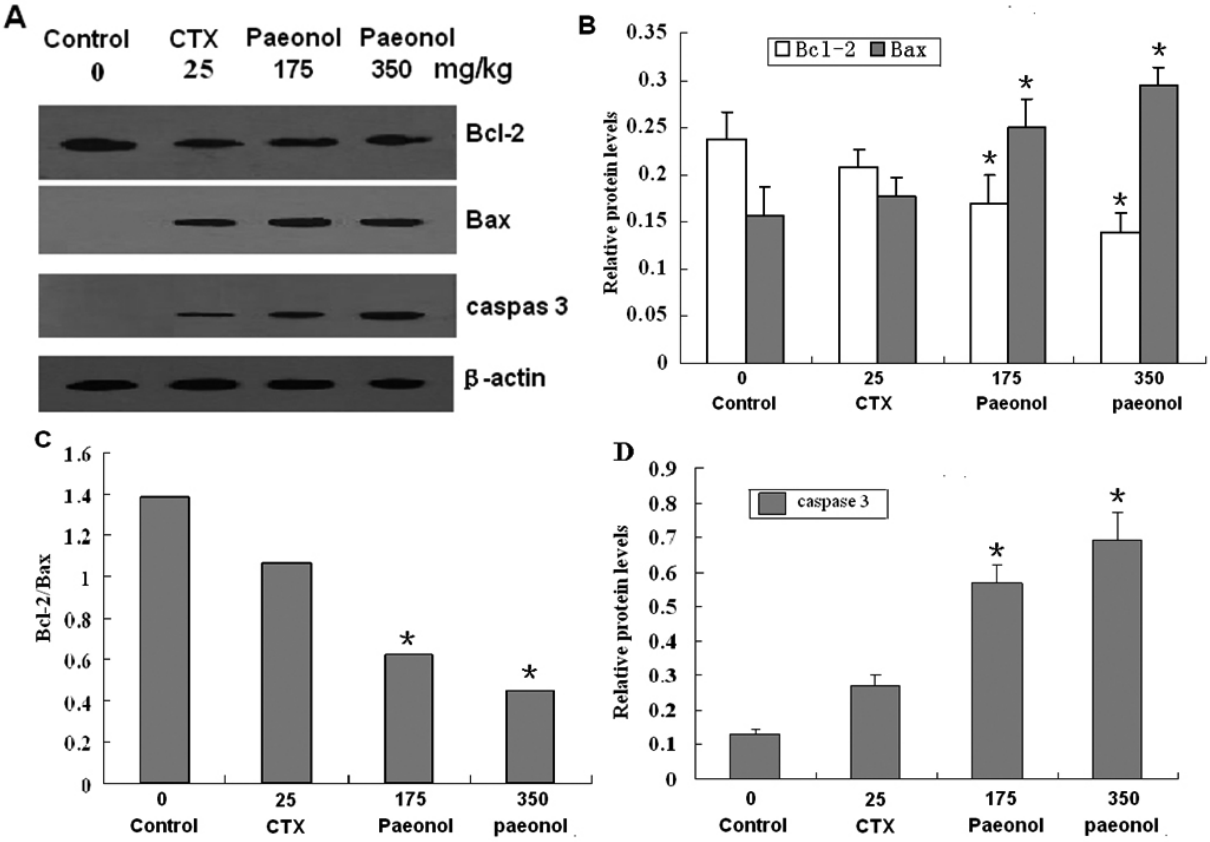

Figure 6. Western blot analysis for Bcl-2, Bax, and caspase-3 in tumor tissue. **A**: Bcl-2, Bax and caspase-3 expression by western blot method. β-actin was used as a control; **B**: The intensity of Bcl-2 and Bax bands was quantified and was shown as relative expression level after normalized by β-actin. *P < 0.05, *vs.* control group. **C**: the ratio of Bcl-2/Bax was showed. *P < 0.05 *vs.* control group. **D**: The intensity of caspase 3 was quantified and was shown as relative expression level after normalized by β-actin. P < 0.05, *vs.* control group.

pounds for potential use as effective therapeutic agents for breast cancer is an important undertaking.

Paeonol is identified to have various physiological activities. It had been also reported that paeonol had the anti-tumor effect. However, the underlying mechanism of the anti-tumor property of paeonol has not yet been fully elucidated. Therefore, we need further explored the possible mechanism of paeonol against breast cancer.

In the present study, paeonol exhibits antitumor biochemical activity on mice with EMT6 breast carcinoma in a dose-dependent manner. After administrated with paeonol, Tumor growth was inhibited and tumor cells appeared typical morphological alterations of the apoptosis, including reduction in cell volume, chromation condensation, deformed and fragment nuclei, and so on. Furthermore, after paeonol treatment, apoptotic peak (Sub-G1 phase), which resulted from the internucleosomal degradation of DNA appeared before G1 phase, These results indicated that inhibition effects of paeonol on breast cancer mice was through inducing tumor cell apoptosis.

Apoptosis is one of the types of programmed cell death, which enables organisms to eliminate malignant cells that threaten survival. Many anticancer drugs may induce tumor cell apoptosis and the induction of apoptosis in tumor cells is generally regarded as a valid measure for anticancer therapy. The mechanisms of apoptosis induced by drugs are complex because of the differences in cell types and drugs [14]. However, mitochondrial and cell-surface death receptor mediated apoptosis are the two principal pathways. The mitochondrial pathway is thought to play a major role in response to cancer treatments and is mediated by the Bcl-2 family proteins, which are always over-expressed in many tumor cells [15-17]. Bcl-2 family proteins are act as repressors of apoptosis by blocking the release of cytochrome-c, whereas pro-apoptotic members (Bax) act as promoters. These effects are

more dependent on the balance between Bcl-2 and Bax than on Bcl-2 quantity alone [18-19].

Many researches have shown that paeonol induced apoptosis in human gastric cancer cell lines and esophageal cancer cell lines by down-regulating the expression of Bcl-2 and regulating the activity of caspase-3; induced cell death involving apoptosis by regulation of apoptosis related-gene Bcl-2 and Bax expression in mouse HepA-hepatoma and human colon cancer cell lines [5,20-22]. These suggested that one of apoptotic mechanisms induced by paeonol was to trigger the mitochondrial-dependent pathway and associated with caspase-3 activation. So, in this study, the expressions of Bcl-2 family and caspase-3 were examined. The results indicated that paeonol decreased the expression of Bcl-2 and increased the expression of Bax and followed by the activation of caspase-3. The up-regulation of Bax expression and the reduction of Bcl-2 expression in the treated groups led to a decrease in the ratio of Bcl-2/Bax, which might be responsible for the drug-induced apoptotic processes. Caspase-3 is an executioner caspase of apoptosis pathway [23-24]. In the current study, after administration with paeonol there was a considerable increase in caspase-3 protein levels, which indicated that paeonol also promoted caspase-3 protein expression. These results suggested that the apoptosis-induction effect of paeonol might occur through the mitochondrial-dependent pathway, finally leads to the activation of the caspase-3, and eventually lead to apoptosis.

CONCLUSION

The results of the study suggest that paeonol result in the growth inhibition of tumor cells by inducing apoptosis, and its mechanisms likely occur through the triggering of the mitochondrial-dependent pathway and caspase-3 activation. Paeonol, as traditional natural plant compounds, may be a novel chemotherapy against breast cancer.

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