Effects of Osthole on the Proliferation, Cell Cycle and Apoptosis in Human Breast Cancer Cells

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Abstract: Osthole, a natural coumarin derivative, is extracted from the fruit of *Cnidium monnieri* (L.) Cusson, a therapeutic agent in traditional Chinese medicine. Breast cancer is one of the most commonly diagnosed cancers and the leading cause of death in women, in developed and increasingly in developing countries. The objective of this study is to investigate the effects of Osthole on the proliferation, cell cycle and apoptosis in human breast cancer cells. Antiproliferative activity of Osthole was measured with the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Flow cytometry was performed to observe cell cycle distribution and apoptotic body appearance. Our results show that Osthole is effective in inhibiting cell proliferation, promoting G1 phase arrest and inducing apoptosis in breast cancer cells. These findings indicate a need for further evaluating Osthole as an antitumor agent to treat breast cancer patients.

Key words: Osthole; breast cancer; proliferation; cell cycle; apoptosis
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蛇床子素对人乳腺癌细胞增殖、细胞周期及凋亡的影响

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[摘要] 蛇床子素是从传统的中药蛇床子的果实中提取的香豆素类衍生物. 在发达国家以及发展中国家, 乳腺癌是发病率和致死率较高的女性肿瘤之一. 本研究的目的是调查蛇床子素对人乳腺癌细胞的增殖、细胞周期以及凋亡的影响. 蛇床子素的抗细胞增殖活性用 MTT 法测定. 细胞周期的分布以及细胞凋亡用流式细胞术测定. 本研究结果表明, 蛇床子素对抑制乳腺癌细胞的增殖、促进 G1期阻滞以及诱导细胞凋亡有明显作用. 该结果提示有必要进一步研究和评估蛇床子素在乳腺癌治疗中的作用.

[关键词] 蛇床子素, 乳腺癌, 增殖, 细胞周期, 凋亡

Breast cancer is one of the most commonly diagnosed cancers and the leading cause of death in women in developed countries and increasing in developing countries[1]. With 1 million new cases diagnosed in the world annually, it accounts for 18% of all female malignancies[2]. Despite its pervasive impact, the etiology of breast

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cancer and the mechanism that promotes its progression are not fully understood. Although lumpectomy, radiation therapy and hormone therapy have been used for curing breast cancer patients[3,4], recurrence and metastasis are major problems in breast cancer therapy. Therefore, identifying novel drugs for breast cancer is imperative.

Osthole (7-methoxy-8[3-methylpent-2-enyl] coumarin), a natural coumarin derivative (Fig. 1), is extracted from a Chinese herb *Cnidium monnieri* (L.) Cuss which possesses a variety of pharmacological properties[5,6]. Many pharmacological properties of Osthole have been reported, including antidiabetic activity[7], anti-inflammatory activity[8], antinociceptive activity[9] and antitumor activity[10-14]. However, to date, the effect of Osthole on breast cancer cells has not been studied.

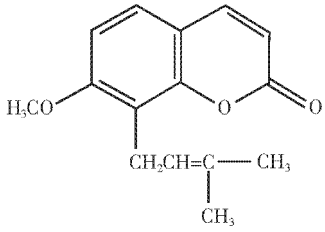

Fig.1 Chemical structure of Osthole
图 1 蛇床子素的化学结构

The present study was performed to investigate the effects of Osthole on cell growth, cell cycle and apoptosis in breast cancer cells.

1 Materials and Methods

1.1 Chemicals

Osthole was obtained from Jiangsu Institute for Food and Drug Control (Nanjing, China).

1.2 Cell culture

Human breast cancer cell line MCF-7 was obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM medium (GIBCO, USA), supplemented with 10% FBS (The Cell Culture Company, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37℃.

1.3 Cell Proliferation assay

Proliferation of MCF-7 cells following treatment with Osthole was measured using 3, 4-(4, 5-dimethylthiazol-2-yl)-2. 5-diphenyltetrazolium bromide (MTT) (Sigma). Briefly, MCF-7 cells were seeded at a density of 8 000 cells/well in a 96-well plate, allowed to adhere overnight and then treated with various concentration of Osthole (0, 20, 40, 60, 80, 100 μmol/L). The cells without Osthole treatment were used as control. At 36 or 48 h after treatment, 20 μL of MTT dye solution was added to each well, and the plate was incubated for 4 h at 37℃. Solubilization/stop solution (150 μL) was added and incubated for 1 h at 37℃ and the absorbance at 570 nm was recorded using a Synergy 8HT plate reader (Bio-Tek Instruments, Winooski, VT).

1.4 Cell cycle and apoptosis analysis

36 h after treatment, the cells were trypsinized and resuspended in 70% ethanol. After incubation on ice for at least 1 h, the cells were resuspended in 1 mL of cell cycle assay buffer (0.38 mmol/L sodium citrate, 0.5 mg/mL RNase A, and 0.15 mg/mL propidium iodide) at a concentration of 5×10^5 cells/mL. Cell cycle analysis was carried out by use of FACS Calibur BD® flow cytometer.

To estimate the number of apoptotic cells, cells were fluorescently labeled by addition of 20 μL of binding buffer, 5 μL of Annexin V-FITC and 5 μL of propidium iodide (BD Pharmingen, San Diego, CA). After the incubation at room temperature in dark for 15 min, cells were applied to flow cytometry analysis.

1.5 Statistical analysis

Results were reported as means ± SD, and statistical analysis was obtained using an unpaired t-test.

2 Results

2.1 Osthole inhibits the proliferation of MCF-7 cells

To examine the effect of Osthole on the proliferation of MCF-7 cells, we performed MTT assay. As shown in Fig. 2, after 36 and 48 h treatment, Osthole significantly inhibited the proliferation of MCF-7 cells in a dose-de-

pendent manner compared with the controls ($P < 0.05$). The growth of MCF-7 cells was inhibited by 12%, 15%, 23%, 31% and 36% with the treatment of Osthole (20, 40, 60, 80, 100 μmol/L) for 36 h. The inhibition rate of MCF-7 cell growth was 27%, 35%, 46%, 52% and 69%, respectively with the treatment of Osthole(20, 40, 60, 80, 100 μmol/L) for 48 h (Fig. 2).

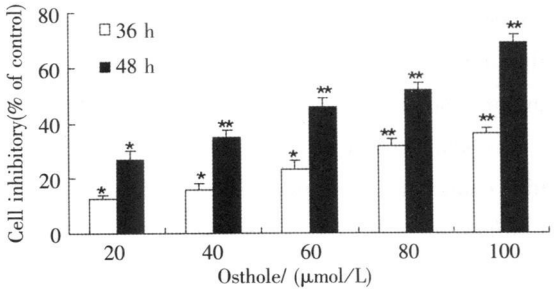

Fig.2 Effects of Osthole on the proliferation of MCF-7 Cells

图 2 蛇虫子素对 MCF-7 细胞增殖的影响

MCF-7 cells were treated with 0, 20, 40, 60, 80 and 100 μmol/L Osthole for the indicated periods of time and cell proliferation was examined by MTT assay. Data were represented as mean±SD of three independent experiments ($P<0.05$).

2.2 Osthole induces cell cycle arrest in MCF-7 cells

As shown in Fig. 3, the G1 phase cell population of control was 40.89%. After 36 h of growth, the G1 phase cell population of Osthole-treated cells increased significantly in a dose-dependent manner. Treatment with 100 μmol/L Osthole led to G1 phase accumulation by 83.87%, and the G1 phase accumulation was accompanied by a corresponding reduction in the percentages of cells in S phase (Fig. 3 and Table 1).

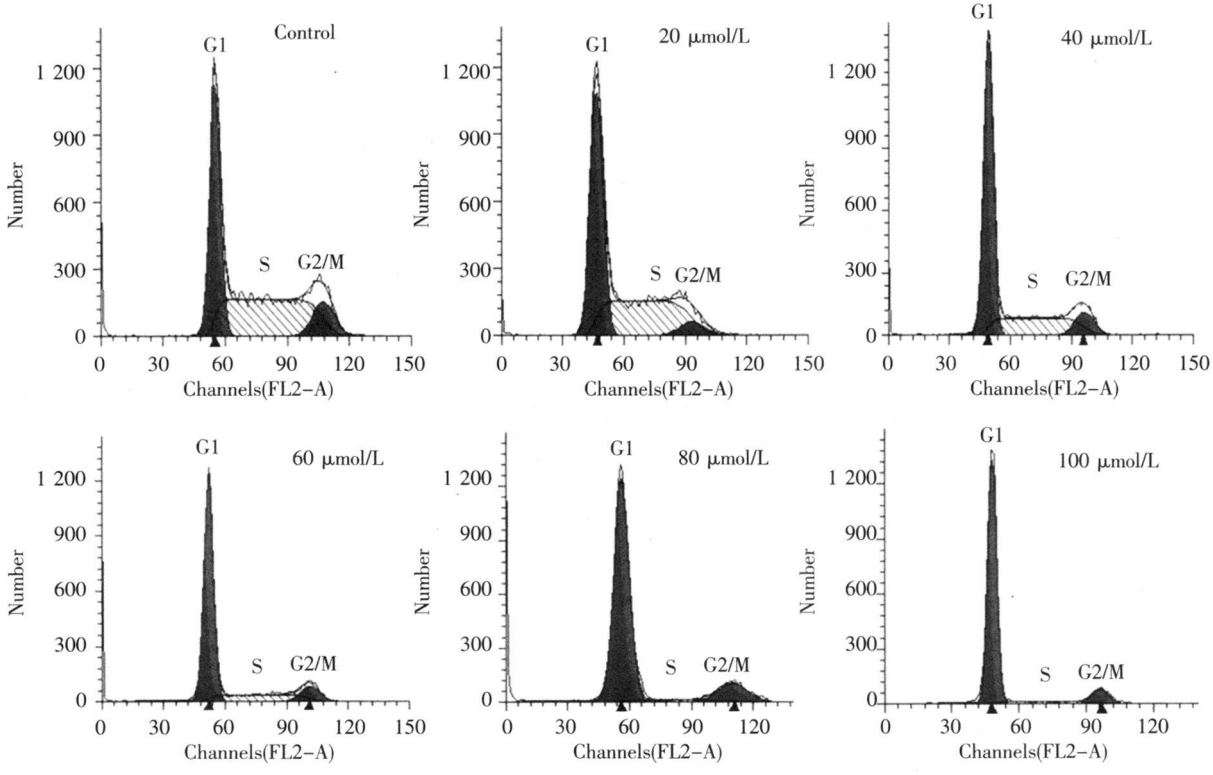

Fig.3 Effects of Osthole on cell cycle of MCF-7 cells

图 3 蛇床子素对 MCF-7 细胞周期的影响

MCF-7 cells were treated with 0, 20, 40, 60, 80 and 100 μmol/L Osthole for 36 h and cells were prepared for the cell cycle detection using flow cytometer. Results are shown for one of three separate experiments.

Table 1 Percentage of MCF-7 cells in G1, S and G2/M phase after Osthole treatment

表 1 蛇床子素处理后, MCF-7细胞的 G1期、S期和 G2/M期细胞的百分比

MCF-7 cells	Control	20 μmol/L	40 μmol/L	60 μmol/L	80 μmol/L	100 μmol/L
G1-Phase/%	40.89±3.6	50.95±2.1	62.10±5.01	72.52±3.6	82.44±4.83	83.87±3.78
S-Phase/%	48.12±2.3	43.34±2.59	28.29±2.96	17.59±3.4	3.92±0.73	6.50±0.96
G2/M-Phase/%	10.99±0.39	5.71±1.1	9.61±1.38	9.89±1.64	13.64±1.88	9.63±2.09

2.3 Osthole induces apoptosis in MCF-7 cells

The number of viable MCF-7 cells decreased following treatment with Osthole. To determine whether cells were undergoing apoptosis in addition to cell cycle arrest, changes in phosphatidylserine membrane localization were analyzed by use of annexin V. As shown in Fig. 4A and B, populations of apoptotic cells were increased in a does-dependent manner with the treatment of Osthole for 36 h.

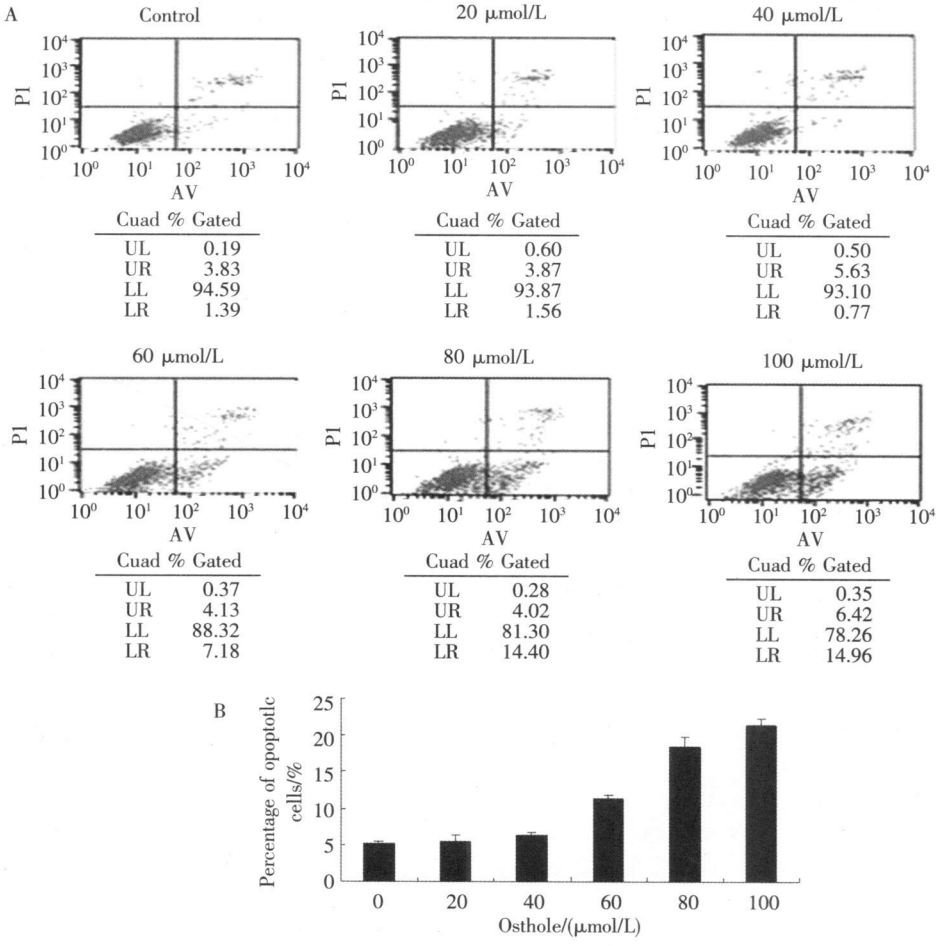

Fig.4 Effect of Osthole on apoptosis of MCF-7 cells

图 4 蛇床子素对 MCF-7 细胞凋亡的影响

MCF-7 cells were treated with 0, 20, 40, 60, 80 and 100 μmol/L Osthole for 36 h. Apoptotic cells were analyzed with Annexin V-FITC using flow cytometer. A: Representative histograms of three separate experiments. B: Results of three separate experiments are shown in graph form.

3 Discussion

Osthole displayed an anti-proliferation function in rat vascular smooth muscle cells, human prostatic cancer cells and human gastric adenocarcinoma, etc[11, 14, 15]. Our results of MTT assay showed that the proliferation of MCF-7 cells was reduced with Osthole treatment in a dose dependent manner (Fig. 2). The inhibition rate for MCF-7 cell growth was 36% with Osthole treatment at 100 μmol/L for 36 h, while the inhibition rate was 69% with Osthole treatment at 100 μmol/L for 48 h. These results suggest that the proliferation of MCF-7 cells was inhibited by Osthole in a time dependent manner.

We performed flow cytometry analysis to characterize the underlying mechanisms by which Osthole inhibits cell proliferation. Upon treatment with 100 μmol/L Osthole for 36 h, the proportion of cells in the G1 phase increased to 83.83%, being remarkablely higher than that of the control (40.89%) (Fig. 3). Therefore, G1 phase arrest may be one of the causes which lead to the inhibition of MCF-7 cell proliferation by Osthole. Result of flow cytometry analysis also showed that Osthole induces apoptosis of MCF-7 cells (Fig. 4A and B). How-

er, the mechanisms by which Osthole induces apoptosis and cell cycle arrest in breast cancer cells need to be studied further. The effects of Osthole on the proteins which are involved in apoptosis such as inhibitor of apoptosis proteins (IAPs), Bcl-2 family members, cytochrome-c release from mitochondria, changes of mitochondrial membrane potential, Fas receptor expression, caspases, p53, as well as cell cycle regulators such as protein Rb, cdk4, Cyclin E, Cydin D1, cdk2, etc will be investigated in the future study.

In summary, Osthole, as an active compound of *Cnidium monnieri* (L.) Cusson, has been reported to posses several biological functions. However, its effects on breast cancer cells have not been studied. Here, we show that Osthole is effective in inhibiting cell proliferation, inducing cell cycle arrest and apoptosis in breast cancer cells. Because of Osthole's diverse pharmacological activities, it has been proposed the possibility of its development as a promising lead compound for drug discovery[16]. Our results provide some of the molecular basis for further evaluating Osthole as an antitumor agent to treat breast cancer patients.

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