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## Luteolin Suppresses Teratoma Cell Growth and Induces Cell Apoptosis via Inhibiting Bcl-2

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Luteolin, which is found in plant foods, has a range of therapeutic applications. In order to examine the potential roles of luteolin in ovarian teratocarcinoma, the human ovarian teratocarcinoma cell line PA-1 was selected for functional experiments in vitro and in vivo. We demonstrated that luteolin inhibited the proliferation and colony formation of PA-1 cells in vitro. The flow cytometry results suggested that luteolin induced apoptosis of PA-1 cells in a dose-dependent manner. Immunofluorescence and qRT-PCR results showed that the expression of B-cell lymphoma-2 (Bcl-2) was decreased in luteolin-treated cells, whereas the expression of Bcl-2-associated X (Bax) was increased compared with that in the control group. In addition, luteolin inhibited the tumor growth of ovarian teratocarcinoma cells in a xenograft model. All the results suggested that luteolin induced cell apoptosis and inhibited tumor growth of PA-1 cells.

**Keywords:** Luteolin; PA-1; Apoptosis; Bcl-2

### INTRODUCTION

Ovarian cancer can be grouped by cellular origin, including epithelial cells (ovarian carcinoma), stromal cells (ovarian adenoma), and germ cells (ovarian teratoma and teratocarcinoma)<sup>1</sup>. Ovarian teratocarcinoma mainly consists of various layers of differentiated cells and embryonal carcinoma stem cells<sup>2</sup>. The main current management of ovarian teratocarcinoma is surgery in combination with chemotherapeutics<sup>3</sup>. In general, all patients with malignant ovarian teratoma will have the same staging surgery that is done for patients with epithelial ovarian cancer<sup>4,5</sup>. Tumor cell metastasis is one of the main causes of death in patients with cancer. The metastatic process is a complex and multistage process, which includes invasion of cancer cells into surrounding tissue, entrances into lymphatic and/or blood vessels, departures from the vessels, and growth at a distant tissue<sup>6</sup>. Therefore, inhibition of cancer cell migration and invasion is considered to be the vital strategy for combating cancer metastasis.

PA-1 cells, which are derived from the ovary, are a well-characterized human ovarian teratoma cell line<sup>7</sup>. This cell line has several malignant characters, which

are similar to embryonic stem cells, including morphological, antigen expression patterns, biochemical, and developmental potentials. The use of natural products as a source of therapeutic agents is important in anticancer therapies<sup>8</sup>. Flavonoids are universally found in fruits, vegetables, and plant-based foods, such as olive oil, tea, and red wine<sup>9</sup>. They also possess broad therapeutic effects such as antiviral, hypoglycemic, anti-inflammatory, and neurological protection. Luteolin, which belongs to the flavonoids, can be extracted from many natural plants such as greens, fruit crops, and medicinal herbs<sup>10</sup>. Luteolin possesses a variety of pharmacological activities as do other flavonoids, including antioxidants, antineoplastic, antiphlogistic, heart protection, and immunomodulatory effects<sup>11,12</sup>. Among all the characteristics, anticancer activity has aroused widespread interest. Previous studies have demonstrated that luteolin has anticancer activities in various types of cancers, such as apoptosis induction, cell cycle arrest, and metastasis inhibition<sup>13,14</sup>. The cytostatic activity of luteolin, which results from the induction of cell cycle arrest, is dependent on the G<sub>1</sub>/S or G<sub>2</sub>/M cell cycle checkpoint<sup>15,16</sup>. Nevertheless, the antitumor activity of luteolin on human ovarian teratoma cells in vitro and

in vivo remains unknown. Hence, the inhibitory effect of luteolin on PA-1 cells in vitro was investigated, and the potential anticancer effects of luteolin in teratocarcinoma xenograft mouse models were also investigated.

## MATERIALS AND METHODS

### Cell Culture

The human ovarian teratoma cell line PA-1 was acquired from Shanghai cell bank (China Academy of Science) and was cultured with DMEM (Thermo Fisher Scientific, Beijing, P.R. China) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 µg/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C.

### 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay

PA-1 cells ( $3 \times 10^4$ ) were seeded into a 96-well plate. Cells were treated with 0, 10, 20, 40, and 80 µM luteolin for 72 h. Then 100 µl of the MTT solution was added to each well, and cells were cultured for 4 h at 37°C. The absorbance was measured at 450 nm using Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA).

### Colony Formation Assay

Single PA-1 cell suspension was seeded in six-well plates and treated with luteolin (10, 20, and 40 µM) for 21 days. The colonies were then fixed using 4% paraformaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich). The number of visible colonies (>50 cells) was counted.

### Cell Apoptosis Analysis

The apoptosis of PA-1 cells was analyzed using Annexin-V-FITC/PI apoptosis kit (Keygen Biotech Co., Ltd, Nanjing, Jiangsu, P.R. China). PA-1 cells were treated with luteolin (0, 10, 20, and 40 µM) for 72 h. Then cells were washed twice with PBS and stained with 5 µl of annexin V-FITC and 5 µl of PI in 500 µl of binding buffer for 15 min at room temperature in the dark. The apoptotic cells were determined using BD FACS Diva software (BD Biosciences, Franklin Lakes, NJ, USA). Annexin V staining serves as a measure of phosphatidylserine externalization, and cells that are annexin V<sup>+</sup>/PI<sup>+</sup> represent early apoptotic cells.

### Immunofluorescence Assay

PA-1 cells were fixed by pre-cold acetone and then rinsed three times with PBS. The cells were permeabilized in 0.1% Triton X-100 and incubated with 1% BSA/PBS. Subsequently, the cells were immunostained

by incubating with rabbit monoclonal antibody against B-cell lymphoma-2 (Bcl-2) or Bcl-2-associated X (Bax; 1:500; Epitomics) overnight at 4°C. After being washed with PBS, cells were incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:100; Boster Biotechnology, Wuhan, Hubei, P.R. China). Nuclei were stained with DAPI (Biotime Biotech, Haimen, Jiangsu, P.R. China). Images were taken on a Zeiss invert microscope (CarlZeiss, Hallbergnos, Germany).

### Quantitative Real-Time PCR (qRT-PCR) Assay

Total RNA was extracted from PA-1 cells using RNAprep Pure Cell/Bacteria Kit (TianGen Biotech, Beijing, P.R. China). First-strand cDNA was synthesized with 1 µg of total RNA using a PrimeScript RT reagent kit (TakaraBio, Tokyo, Japan). qRT-PCR was performed using IQ™ SYBR Green supermix and the iQ5 real-time detection system (Bio-Rad Laboratories, Hercules, CA, USA). The comparative cycle threshold (Ct) method was applied to quantify the expression levels through calculating the  $2^{-\Delta\Delta C_t}$  method. The primers used for PCR were as follows: GAPDH: 5'-AAGGACCTGTATGCCAACACA-3' and 5'-ATCCACACAGAATACTTGCGTT-3; Bcl-2: 5'-CACAAGAGGCCAAGGCTACCT-3' and 5'-CAGGAAAGCAGGAAGTCTCAA-3'; Bax: 5'-ATTGAGAAACGATTTGCCCTACA-3' and 5'-GGGAAATGGCTTATTCTCCTTTGCTT-3'.

### Xenograft Model

For the xenograft model, 100 µl of PA-1 cells ( $5 \times 10^6$ ) was subcutaneously inoculated into BALB/c nude mice. Mice were randomly assigned to three groups: control group, 10 mg/kg luteolin-treated group, and 20 mg/kg luteolin-treated group. Luteolin was dissolved in DMSO. Mice in the experimental groups were administered luteolin intragastrically daily for 28 days. Control mice in vehicle-treated group received the same dose of vehicle (DMSO). Tumor width (*W*) and length (*L*) were measured each week. The tumor volume was calculated using the formula:  $L \times W^2/2$ . After 28 days, mice were sacrificed. Briefly, formalin-fixed paraffin-embedded tumor tissues were prepared in 4-µm sections, and Ki-67 immunohistochemical (IHC) staining assay was performed. The number of immunopositive cells was counted using the BZ-9000 analysis software programmer (Keyence, Osaka, Japan).

### Statistical Analysis

Statistical analysis was performed using SPSS 19.0 software. Results were presented as means ± standard deviation (SD). The difference was determined by Student's *t*-test, and a value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### *Luteolin Inhibits the Proliferation of PA-1 Cells In Vitro*

Luteolin (Fig. 1A) is a natural flavonoid that can be extracted from greens, fruits, and herbal medicines. As shown in Figure 1B, the MTT assay suggested that luteolin suppressed the growth of PA-1 cells in a dose-dependent and time-dependent manner compared to the control group. To determine the effects of luteolin on the colony formation of PA-1 cells, the colony formation assay was performed. As shown in Figure 1C, the colony formation of PA-1 cells was significantly inhibited by luteolin. Moreover, the morphological changes were also observed in PA-1 cells treated with luteolin for 72 h. As shown in Figure 1D, the number of dead cells and cell fragments was increased by luteolin in a dose-dependent manner.

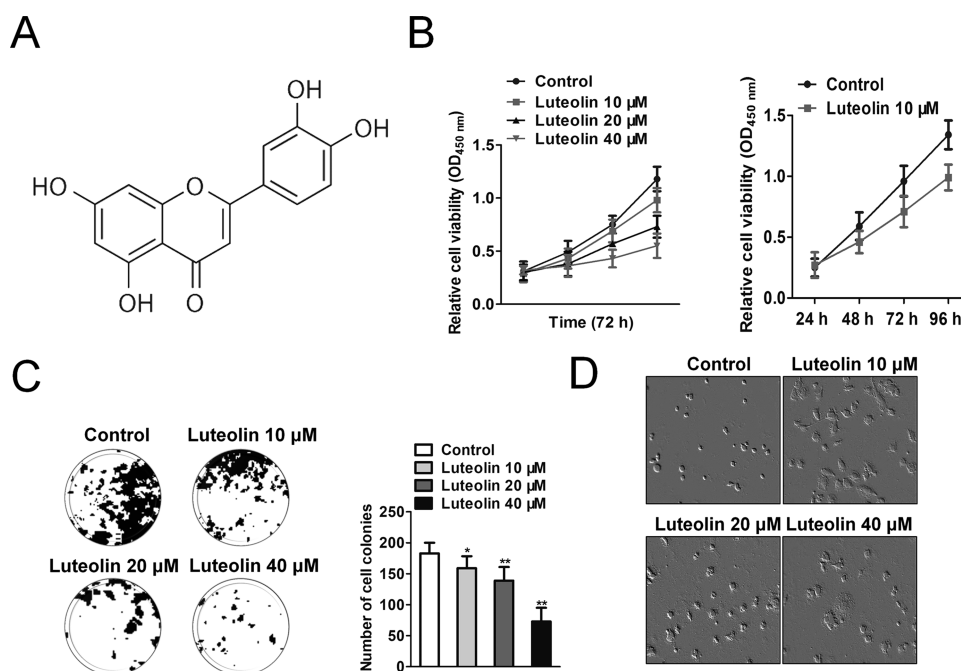
### *Luteolin Induces the Apoptosis of PA-1 Cells*

To further explore the effects of luteolin on the apoptosis of PA-1 cells, cells were stained with annexin V-FITC/PI, and the apoptosis was analyzed by flow cytometry. The apoptotic rate of PA-1 cells was significantly increased by luteolin (10–40  $\mu$ M). Representative results are shown in Figure 2A. As shown in Figure 2B, following

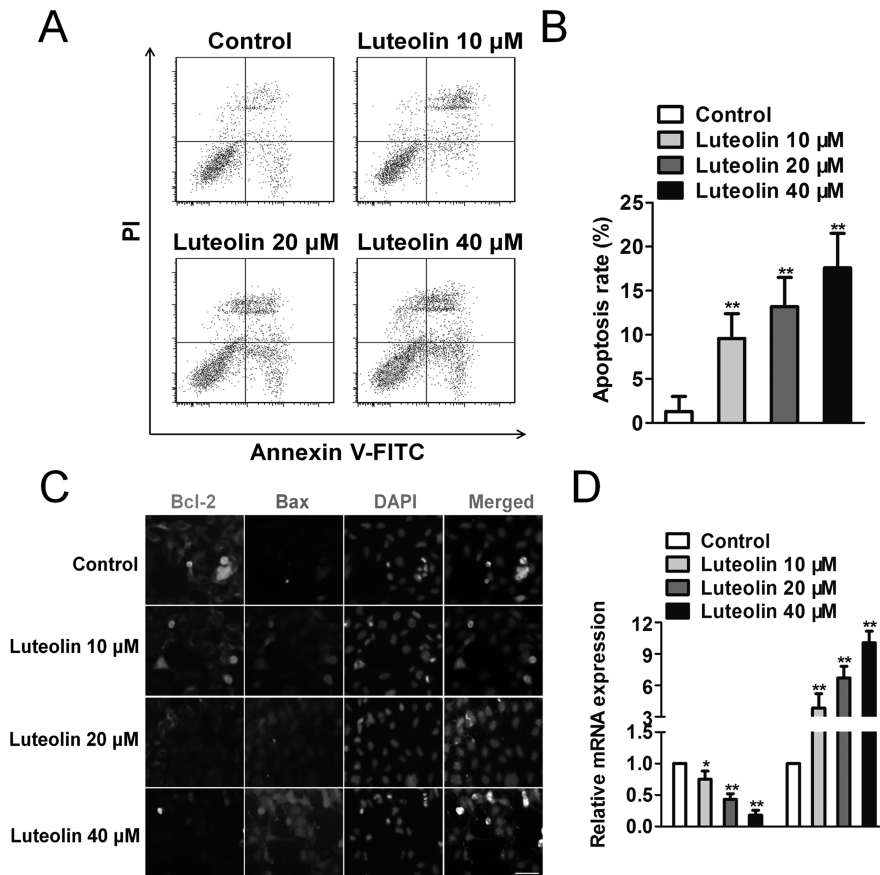
treatment with 10, 20, and 40  $\mu$ M luteolin, the percentage of apoptotic cells was  $12.44 \pm 0.43\%$ ,  $19.77 \pm 0.83\%$ , and  $38.71 \pm 0.7\%$ , respectively, which was much higher than that in the control group ( $4.31 \pm 0.2\%$ ). Bcl-2 is considered an antiapoptotic protein, whereas Bax is considered a proapoptotic gene. To detect the expressions of Bcl-2 and Bax, immunofluorescence analysis was performed. Following treatment with luteolin (10–40  $\mu$ M) for 48 h, the expression of Bcl-2 was decreased, whereas the level of Bax was increased compared with the control group (Fig. 2C). Consistently, compared with the control group, the treatment of luteolin significantly decreased the mRNA level of Bcl-2 and markedly increased the mRNA level of Bax (Fig. 2D).

### *Effect of Luteolin on the Migration and Invasion of Teratocarcinoma Cells*

To examine the influence of luteolin on the migration and invasion of PA-1 cells in vitro, wound healing and Transwell invasion experiments were performed. In wound healing analysis, the wound closure in PA-1 cells treated with different concentrations of luteolin was markedly inhibited compared with that in the control (Fig. 3A). The Transwell result indicated that the invasion of PA-1 cells after luteolin treatment was remarkably



**Figure 1.** The proliferation of PA-1 cells is inhibited by luteolin. (A) The structure of luteolin. (B) PA-1 cells were treated with luteolin (0, 10, 20, and 40  $\mu$ M) for 72 h, and the cell viability was detected using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-h-tetrazolium bromide (MTT) assay (left). PA-1 cells were treated with 10  $\mu$ M luteolin for 24, 48, 72, or 96 h, respectively, and the cell viability was measured the using MTT assay (right panel). (C) Colony formation analysis of PA-1 cells treated with luteolin (0, 10, 20, and 40  $\mu$ M). (D) After treatment with luteolin, the representative morphological changes of PA-1 cells were observed under the invert microscope. \* $p < 0.05$  versus the control group, \*\* $p < 0.01$  versus the control group.



**Figure 2.** Luteolin induces the apoptosis of PA-1 cells. (A) PA-1 cells were treated with 10–40  $\mu\text{M}$  luteolin for 48 h, and apoptosis of cells was detected using flow cytometry. (B) The percentage of apoptotic cells is shown. \*\* $p < 0.01$  versus the control group. (C) PA-1 cells were treated with luteolin, and the expressions of Bcl-2 and Bax were assessed using immunofluorescence analysis. (D) The mRNA levels of Bcl-2 and Bax were determined by quantitative real-time (qRT)-PCR analysis. GAPDH was used as a loading control. \* $p < 0.05$  versus the control group, \*\* $p < 0.01$  versus the control group.

reduced (Fig. 3B). Altogether, these findings suggested that luteolin inhibited migration and invasion of PA-1 cells in vitro.

#### *Luteolin Inhibits the Growth of Teratocarcinoma Cells in a Xenograft Model*

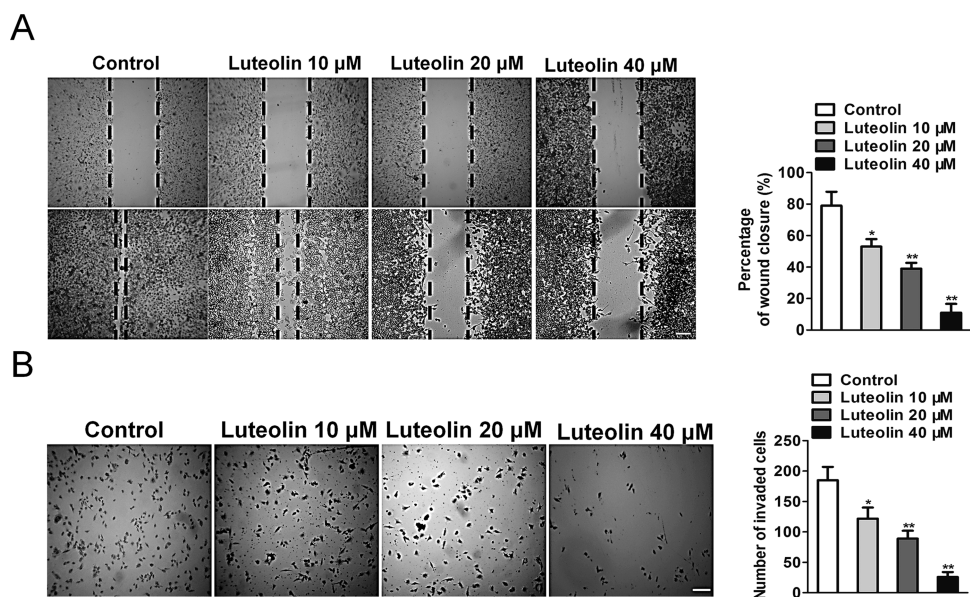
In order to further investigate whether luteolin inhibited the growth of teratogenic cells in vivo, PA-1 cells were inoculated into nude mice. Upon tumor tissue grown to 100  $\text{mm}^3$ , mice were treated with 10 mg/kg or 20 mg/kg of luteolin. The results showed that luteolin notably decreased the tumor volume (Fig. 4A) and tumor weight (Fig. 4B) in nude mice, whereas the body weight was unaffected by luteolin treatment (Fig. 4C). In order to determine the inhibitory effects of luteolin on growth of PA-1 cells in vivo, the expression level of Ki-67 in xenograft tumor tissue was determined. The Ki-67 immunohistochemistry (IHC) staining results revealed that luteolin greatly inhibited the expression of Ki-67 in tumor tissues

(Fig. 4D). These results demonstrated that luteolin inhibited the growth of teratocarcinoma cells in vivo.

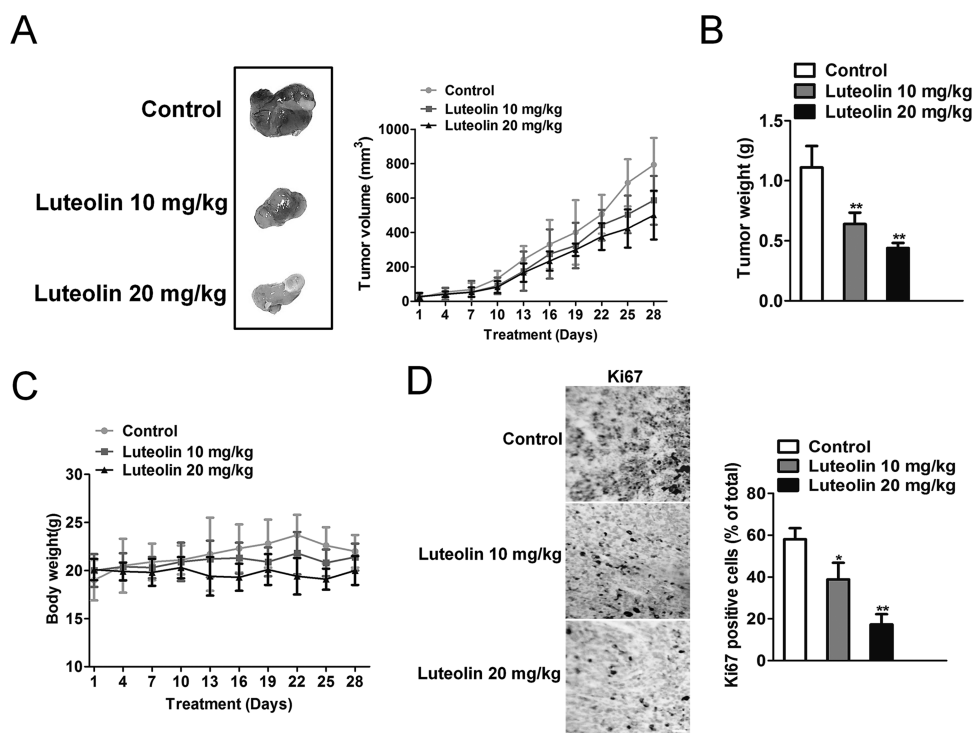
## DISCUSSION

Chemotherapy can be used for the prevention of the progression of ovarian teratoma and improve the survival rate of patients with ovarian teratoma<sup>17</sup>. Cisplatin and oxaliplatin are cytotoxic agents that are mainly used for chemotherapy. However, cytotoxic chemotherapy is a potential danger for clinical side effects<sup>18</sup>. Previous investigations suggested that luteolin could induce cell cycle arrest, inhibit the proliferation, and promote the apoptosis of cancer cells, such as hepatocellular carcinoma, cholangiocarcinoma, and thyroid carcinoma cell in vitro<sup>19</sup>.

It was indicated in our current study that luteolin has an antiproliferation effect on PA-1 ovarian teratoma cells. Results from MTT assay revealed that luteolin was able to inhibit the growth of PA-1 cells in a dose-dependent manner in vitro as well as suppress the colony formation



**Figure 3.** Luteolin treatment reduces the migration and invasion of PA-1 cells. (A) PA-1 cells were treated with 10–40  $\mu$ M luteolin, and the migration was determined by wound healing assay. Data calculated as the mean  $\pm$  standard deviation (SD),  $n=3$ . \* $p<0.05$  versus the control group, \*\* $p<0.01$  versus the control group. (B) PA-1 cells were treated with 10–40  $\mu$ M luteolin, and the invasion was determined by Transwell assay. Data recorded as the mean  $\pm$  SD,  $n=3$ . \* $p<0.05$  versus the control group, \*\* $p<0.01$  versus the control group.



**Figure 4.** Luteolin inhibits the tumor growth of PA-1 cells in vivo. (A) Representative images of xenograft tumor tissue isolated from luteolin-treated and control groups. (B) Tumor weight was significantly decreased in nude mice treated with luteolin. (C) The body weight in the control mice and the luteolin treatment groups. (D) Ki-67 immunohistochemistry staining using the tumor tissue isolated from luteolin-treated and control groups. \* $p<0.05$  versus the control group, \*\* $p<0.01$  versus the control group.

of PA-1 cells. The flow cytometry results revealed that luteolin treatment induced apoptosis in PA-1 cells. Apoptosis is the process of cellular self-destruction, and genes such as Bcl-2 and Bax are known to inhibit and promote apoptosis. The flow cytometry results demonstrated that the apoptotic rate of PA-1 cells could be increased by luteolin in a dose-dependent manner. The mechanism of Bax/Bcl-2 activation remains a central question in mitochondria-dependent apoptotic signaling. Consistent with flow cytometry, both immunofluorescence and qRT-PCR analysis demonstrated that luteolin reduced the expression of Bcl-1 and increased the level of Bax in PA-1 cells. Tumor metastasis is mainly a cause of death in patients with cancer. Our results also indicated that luteolin inhibited PA-1 cell migration and invasion in vitro.

In addition, PA-1 cells were subcutaneously injected into nude mice to construct the teratocarcinoma xenograft model. The results indicated the mice treated with luteolin had smaller tumor sizes than those in the control group. There were few cytotoxic side effects obviously observed in luteolin-treated mice. This is in accordance with the results from other investigations that luteolin could significantly suppress the cancer growth of non-small cell lung cancer (NSCLC), head and neck squamous cell carcinoma (HNSCC), and breast cancer in xenograft models in vivo<sup>20</sup>. Collectively, in the current study, luteolin could result in PA-1 ovarian teratoma cell apoptosis and also inhibit tumor growth of PA-1 cells in a xenograft model. This study revealed the potential mechanisms of luteolin in the treatment and prevention of ovarian teratoma, and also suggested the potential of luteolin in the treatment for ovarian teratoma.

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