Effects of Quercetin Extracted from Flower Buds of *Sophora japonica* cv. jinhuai on Proliferation and Apoptosis of Human Breast Cancer MCF-7 Cells

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Abstract [**Objectives**] To investigate the effects of quercetin extracted from flower buds of *Sophora japonica* cv. jinhuai on the proliferation, apoptosis and migration of human breast cancer MCF-7 cells. [**Methods**] MTT assay, inverted microscope observation, hoechst33342 staining, flow cytometry (FCM) and wound healing assay were adopted to investigate the proliferation, morphological changes, apoptosis level and cell migration ability of human breast cancer MCF-7 cells, respectively. [**Results**] The morphological changes of cells in the treatment groups included gradually decreased number, reduced volume, vague cell contour, loose intercellular connection, uneven cytoplasm distribution and increased cell debris. With the increase of drug concentration, quercetin significantly inhibited the proliferation of human breast cancer MCF-7 cells ($P<0.05$). The number of apoptotic bodies increased gradually. When the concentration reached 100 μmol/L, a large number of nuclear fragments appeared, and the level of apoptosis was statistically different ($P<0.05$). The mobility and migration ability of cells showed a decreasing trend, and the differences were statistically significant ($P<0.05$). [**Conclusions**] This study can provide experimental basis for clinical application of quercetin against breast cancer.

Key words Quercetin, Apoptosis, Cell migration, Cell proliferation, MTT assay, Anti-breast cancer

1 Introduction

Breast cancer is the second most common cancer diagnosed among women, second only to nonmelanoma skin cancer. It is the main cause of cancer death among women all over the world[1]. Worldwide, breast cancer accounts for about 30% of female cancers, with a mortality rate of 15%, and the incidence rate has increased rapidly in recent decades[2]. Although great progress has been made in screening methods for early diagnosis, breast cancer is still a global threat, and it is still the main cancer diagnosed by women and needs effective treatment. At present, due to the high incidence of tumor recurrence and disease progression after single drug therapy such as surgery, radiotherapy, endocrine therapy and chemotherapy, combined therapy has become a symbol of breast cancer treatment[3], in order to minimize or eliminate recurrence, drug resistance and toxic effects, and ensure that patients have a good quality of life.

According to the data, breast cancer can be divided into two main subtypes: hormone receptor positive /ERBB2 negative, ERBB2 positive and triple negative, and about 75% of breast cancer patients are estrogen receptor (ER)-positive breast cancer[4]. High levels of estrogen in the body can regulate the growth and proliferation of breast cancer cells, suggesting that ER may become a key target for breast cancer treatment. However, patients with ER(+) breast cancer are more likely to show drug resistance than those with ER-negative breast cancer, which leads to a great decrease in the efficacy of anti-estrogen therapy. Therefore, improving the therapeutic effect of breast cancer by finding safer and more efficient anti-estrogen drugs has become one of the research hotspots in the field of new anti-breast cancer drug research and development.

It has been found that leguminous plants are rich in phytoestrogens, and their molecular structures are similar to those in mammals. When combined with ER receptors, they can play an anti-estrogen role and have a certain preventive and therapeutic effect on estrogen-related malignant tumors such as breast cancer and ovarian cancer[6-7]. Quercetin is one of the important phytoestrogens in dry flower buds of *Sophora japonica* cv. jinhuai in northern Guangxi, a characteristic tree species in northern Guangxi[8]. Quercetin is a natural product of flavonols, which has many physiological activities, such as anti-tumor, anti-inflammatory, anti-oxidation, hypoglycemic, body weight-reducing, antihypertensive and antidepressant[9], but the specific mechanism of action is still unclear.

In this study, breast cancer MCF-7 cells and normal breast epithelial cells MCF-10A were used as cell models to investigate the effects of quercetin on the proliferation rates of the two kinds of cells; and the effects of quercetin on the morphology, apoptosis and migration ability of MCF-7 cells were investigated by molecular biology techniques, in order to provide experimental basis for the development of new anti-breast cancer drugs of quercetin, as well as experimental reference for the clinical application of quercetin against breast cancer.

2 Materials and methods

2.1 Materials

2.1.1 Materials and reagents. Quercetin, extracted from flower

Received: March 26, 2024 Accepted: July 1, 2024
Supported by Guilin Scientific Research and Technology Development Program (20210202-1; 20220104-4); Special Project of the Central Government in Guidance of Local Science and Technology Development (ZY20230102).
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buds of *Sophora japonica* cv. jinhuai in northern Guangxi by alkali extraction and acid precipitation, and then purified by silica gel column chromatography and determined by HPLC to have a quercetin content of 96.7%; fetal bovine serum (FBS) (batch No.: 11011-8611), purchased from Hangzhou Sijiqing Co., Ltd.; DMEM (batch No.: 12800-017), PBS powder (batch No. 10010023), RPMI-1640 medium (batch No.: 12633-012) and trypsin powder (batch No.:15400054), all purchased from Gbico, USA; MTT dry powder (batch No.:IM0280) and mixed solution of streptomycin (100 ×) (batch No.: P1400), purchased from Beijing Solarbio Science & Technology Co., Ltd.; fluorescent dye Hoeehst33342 (batch No.:23491-52-3), DMSO (batch No.: A11605), APS (batch number A02053) and TEMED (batch No.: A12843), purchased from AMRESCO, USA; Annexin V apoptosis kit (batch No.: V13242), purchased from eBioscience; human breast cancer cell line MCF-7 and human normal mammary epithelial cell MCF-10A, purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

2.1.2 Instruments. Inverted fluorescence microscope, OLYMPUS IX51, Japan; MLDEL 680 microplate reader, BIO-RAD, USA; flow cytometry, BD, USA; CO_2 incubator, pipettes, frozen high-speed centrifuge and ultra-low temperature refrigerator, all from Thermo Fisher, USA; high performance liquid chromatograph E2695, Waters, USA.

2.2 Experimental methods

2.2.1 Detection of effects of quercetin on proliferation rates of MCF-7 and MCF-10A cells. The effects of quercetin on the proliferation of MCF-7 and MCF-10A cells were detected by MTT assay. The cells were grown in RPMI 1640 complete medium containing 10% fetal bovine serum and 1% streptomycin, and cultured in a cell incubator with 5% CO_2 at 37 ℃ for 2 – 3 d. After the cells reached the logarithmic growth stage, cell suspensions (2.5×10^4 cells/mL) were prepared after digestion. Next, 200 μL was added into each well of a 96-well plate. The cells were divided into a blank group, a normal control group, and treatment groups (20, 50, and 100 μmol/L quercetin). Each group was set with three parallel wells, and the cells were tested at each of following three time points: 24, 48, and 72 h. At 4 h before the detection point, 20 μL of 5 mg/mL MTT solution was added to each well, and after incubating at 37 ℃ for 4 h, the absorbance (*OD*) value was measured at the wavelength of 570 nm using a microplate reader. The inhibition rate (GI) of cell proliferation was calculated: GI (%) = 1 – *OD* value of experimental group/*OD* value of control group.

2.2.2 Observation of effects of quercetin on morphological changes of MCF-7 cells. MCF-7 cells in the logarithmic growth stage were digested, and the cell suspension was adjusted to 5×10^5 cells/mL. Next, the cells were inoculated to 6-well plates. When the fusion degree was about 60%, the culture medium was replaced with new medium containing 0, 25, 50 and 100 μmol/L quercetin, respectively, and culture was continued for 24 h. Finally, the MCF-7 cells were observed for morphological changes under an inverted microscope.

2.2.3 Observation of effects of quercetin on apoptosis morphology of MCF-7 cells by Hoechst33342 staining. For MCF-7 cells in the logarithmic growth stage, the cell suspension was adjusted to 4×10^5 cells/mL and inoculated into 6-well plates. The experimental groups and drug concentrations were set according to Section **2.2.1**. After 24 h of drug action, all cells including suspending cells and those adhering to the wall in each plate were collected, and Hoechst33342 dye solution (1 mL/well) prepared according to the instructions of the reagent was added to the cells, which were then incubated at a constant temperature of 37 ℃ in the dark for 20 min. After removing the Hoechst33342 dye solution, the apoptosis morphology was observed under an inverted fluorescent microscope.

2.2.4 Detection of effects of quercetin on apoptosis level of MCF-7 cells by flow cytometry. MCF-7 cells in the logarithmic growth stage were cultured in 6-well plates. When the fusion degree was about 60%, 2 mL of DMEM medium containing 0, 25, 50 and 100 μmol/L quercetin were given, respectively. After culturing for 24 h, the supernatant and the washing liquid after washing the cells with 3 mL of ice 1 × PBS solution were all transferred to a centrifuge tube. After digesting and re-suspending the cells, the cells were collected according to the instructions of AnnexinV FITC kit, dyed in the dark for 1 h at room temperature, and filtered by a 300-mesh sieve. Finally, the apoptosis rate was analyzed on a computer within 30 min.

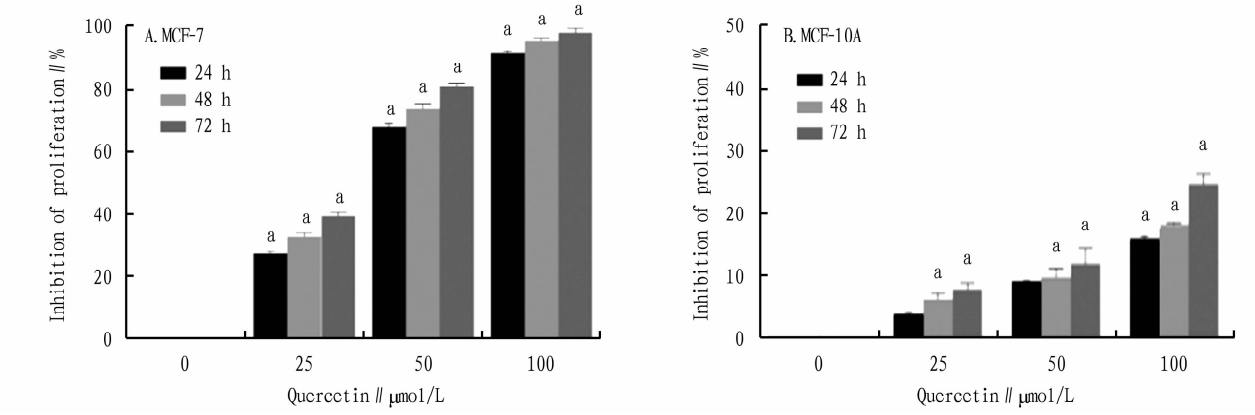
2.2.5 Detection of effects of quercetin on apoptosis level of MCF-7 cells. After drug administration, the cells in each group were incubated at 37 ℃ for 48 h. Next, the cells were fixed with 4% paraformaldehyde for 15 min, and dyed with 1% crystal violet for 15 min. Subsequently, the chambers were cleaned with phosphate buffer, and the cells in the chambers were wiped off with cotton swabs. After fully air-drying, five visual fields were randomly selected under a microscope, and the number of transmembrane cells was analyzed by Image J software, and the cell invasion rate was calculated: Cell invasion rate (%) = Number of transmembrane cells in the model group or the administration group/Number of transmembrane cells in the control group × 100%.

2.2.6 Detection of effects of quercetin on migration of MCF-7 cells. The effects of quercetin on cell migration were detected by a cell scratch assay. Cells in the logarithmic growth stage were digested, and the cell suspension was adjusted to 5×10^5 cells/mL. Next, the cells were inoculated to 6-well plates, and the experimental drug concentrations were set according to the results in section Section **2.2.1**. When the cells completely covered the 6-well plates, a straight scratch was made vertically in the 6-well plates with a 100 μL gun head, and then, the floating cells were washed with 1 × PBS solution. The cells of the three groups were photographed and recorded in the same position under an inverted microscope at 0 and 24 h after drug administration in the administra-

tion groups, and the scratch width was measured to calculate cell migration rate: Cell migration rate (%) = (1 - Scratch width at measurement/Initial scratch width) ×100%.

3 Results and analysis

3.1 Effects of quercetin on proliferation of breast cancer cells MCF-7 and breast epithelial cells MCF-10A Compared with the blank control group, with the concentration of quercetin increasing, the proliferation inhibition rates of MCF-7 cells in different drug groups gradually increased, and the differences were statistically significant ($P < 0.01$). The proliferation inhibition rates of MCF-7 cells in various groups showed a trend of changing with time ($P < 0.01$), and gradually increased with the extension of drug intervention time. The inhibition rate of cell proliferation increased with the increase of drug concentration and the extension of drug action time, that is, there was an interactive effect between drug groups and action time. As for MCF-10A, with the concentration of quercetin increasing, the inhibition rates of cell proliferation in various groups did not increase significantly, and when the quercetin concentration reached 100 μmol/L, the inhibition rate was as high as (24.81 ± 1.51)%, indicating that quercetin had little effect on the proliferation of normal breast epithelial cells MCF-10A. The results are shown in Fig. 1.



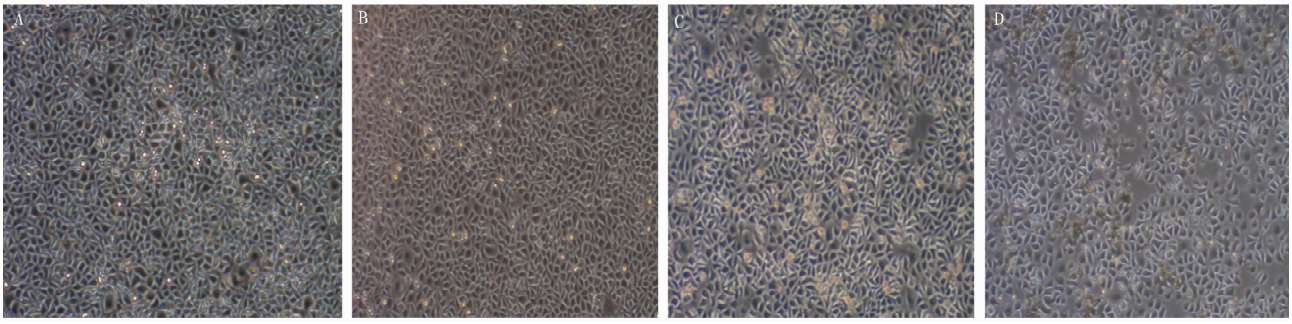
NOTE Compared with the blank control group, $^{a}P < 0.01$; comparing any two concentration groups, $^{*}P < 0.05$.

Fig. 1 Effects of quercetin on the proliferation of MCF-7 and MCF-10A cells (%, $\bar{x} \pm s$, $n = 3$)

3.2 Effects of quercetin on cell morphology of breast cancer cells MCF-7 MCF-7 cells were treated with different concentrations of quercetin (25, 50 and 100 μmol/L) for 24 h, and photographed by an inverted microscope. The results showed that the cells in the blank control group grew densely and irregularly polygonal, with good growth state, full cell size, clear contour, dense intercellular connection and uniform cytoplasm distribution, while in the experimental groups, with the concentration of quercetin increasing, the cell morphology gradually changed. At low concentration (25 μmol/L), the cell density decreased to some extent, the cell contour was blurred, some cells were abnormal in morphology, and the intercellular connection became loose. When the drug concentration was 50 μmol/L, the number of adherent cells decreased significantly, and the cell morphology changed obviously, resulting in shrinkage, rounding and shedding. When the concentration of quercetin was 100 μmol/L, the number of adherent cells was greatly reduced, local "vacuolation" and irregular bending occurred, the cell volume was significantly reduced, and some cells even showed cytolytic changes (Fig. 2). The results showed that the effect of quercetin on the morphological changes of MCF-7 cells was concentration-dependent.

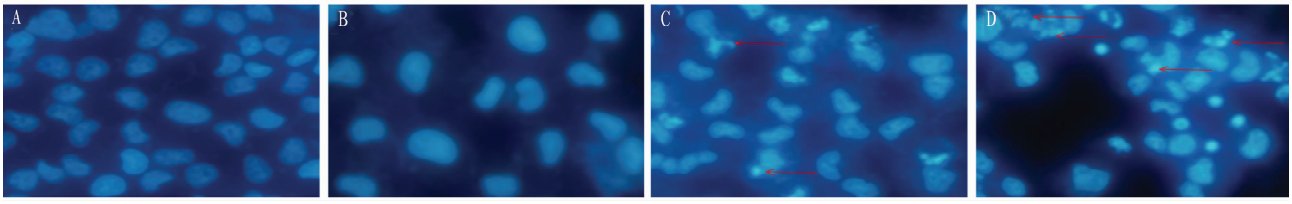
3.3 Effects of quercetin on nuclear morphology of breast cancer cells MCF-7 In the blank group, almost no apoptotic cell was observed in MCF-7 cells, and the nuclei showed regular morphology, with large and complete nucleolus and uniform chromatin distribution. With the concentration of quercetin increasing, the nuclear morphology gradually changed, and the phenomenon of apoptosis became more and more obvious. The volume of apoptotic nuclei was reduced, the chromatin distribution was uneven, and even cell nucleus cracking occurred. In the low-concentration drug group (25 μmol/L), the apoptosis of MCF-7 cells was not significant, and the nuclear structure was basically intact. In the 50 μmol/L drug group, some nuclei changed morphologically, with the chromatin beginning to distribute unevenly, and some nuclei were deformed. When the drug concentration was 100 μmol/L, a large amount of shrunk nuclei could be observed under a light microscope, with highly concentrated chromatin and deepened staining, and the nuclei moved to the edge and cracked into fragments (Fig. 3).

3.4 Effects of quercetin on apoptosis level of breast cancer cells MCF-7 The results showed that the proportion of living cells gradually decreased, and the number of apoptotic and necrotic cells gradually increased with the increase of quercetin concentration. When the concentration of quercetin was 100 μmol/L, the apoptosis rate was the highest. Compared with the blank control group, the differences in apoptosis rate were statistically significant among various drug groups ($P < 0.01$). The results are shown in Fig. 4. Meaning of FCM scatter diagram: In each cell distribution diagram composed of four quadrants, the lower left quadrant represents living cells, the lower right quadrant represents early apoptotic cells, the upper right quadrant represents late apoptotic and necrotic cells, and the upper left quadrant represents cells mechanically damaged during the sampling process.



NOTE A. Blank control group; B. 25 μmol/L quercetin group; C. 50 μmol/L quercetin group; D. 100 μmol/L quercetin group.

Fig. 2 Morphological changes of human breast cancer cells MCF-7 in various groups



NOTE A. Blank control group; B. 25 μmol/L quercetin group; C. 50 μmol/L quercetin group; D. 100 μmol/L quercetin group. The arrows indicate "apoptotic bodies".

Fig. 3 Effects of quercetin on nuclear apoptosis morphology of human breast cancer MCF-7

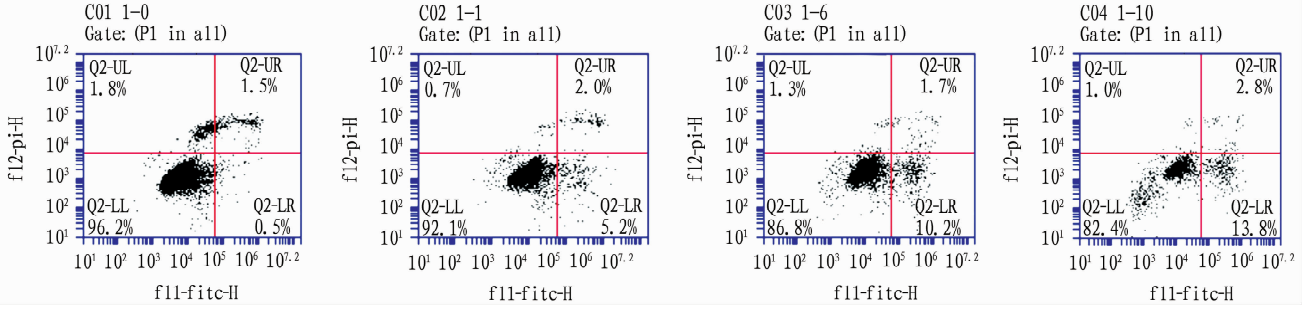


Fig. 4 Effects of quercetin on apoptosis level of human breast cancer MCF-7 cells

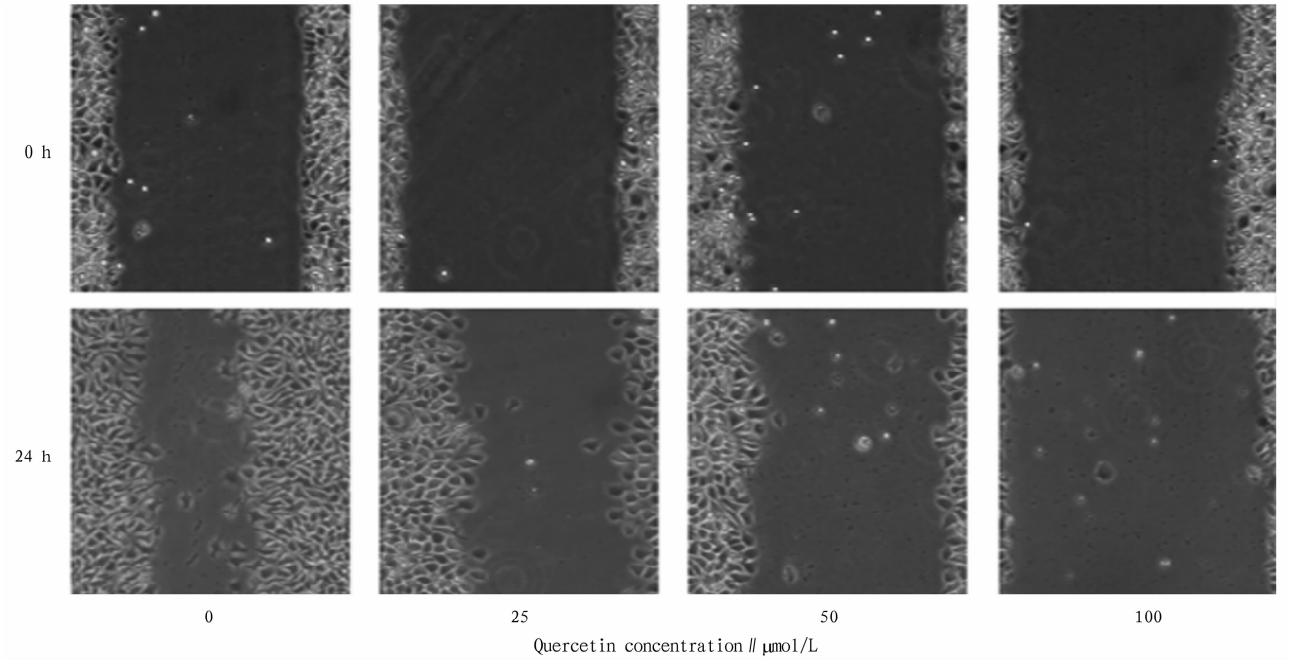


Fig. 5 Effects of quercetin on migration of human breast cancer MCF-7 cells

3.5 Effects of quercetin on migration ability of breast cancer MCF-7 cells With the concentration of quercetin increasing, the healing degree of cell scratches gradually weakened. At 24 h, MCF-7 cells migrated significantly in the scratched area of the blank control group, while the cell healing rates in the scratched areas of the drug treatment groups showed a decreasing trend with the increase of concentration, showing statistical significance ($P < 0.05$). The results are shown in Fig. 5 and Table 1.

Table 1 Effects of quercetin on wound healing rate of MCF-7 cells ($\bar{x} \pm s$, $n = 3$)

Quercetin μmol/L	Cell spacing at 0 h//cm	Cell spacing at 24 h//cm	Healing rate//%
0	0.79 ± 0.003	0.190 ± 0.032	75.97 ± 0.225 *
25	0.93 ± 0.027	0.55 ± 0.012	40.94 ± 1.290[a] *
50	0.85 ± 0.018	0.635 ± 0.019	25.18 ± 1.270[a] *
100	1.06 ± 0.021	0.934 ± 0.045	11.74 ± 2.550[a] *

NOTE Compared with the blank control group, [a]$P < 0.05$; comparing any two concentration groups, *$P < 0.05$.

4 Discussion

Studies have shown that natural products and their derivatives have their unique advantages in the field of cancer prevention and treatment, and plant active components, especially phytoestrogens, can inhibit growth and induce apoptosis in breast cancer[10]. Clinical studies have found that cyclophosphamide alone is not effective in the treatment of advanced bladder cancer, and cyclophosphamide itself is very toxic, resulting in many adverse reactions to normal tissues and poor patient tolerance. When combined with quercetin, it was found that its toxic effect was minimized and the fatigue behavior of patients could be improved. The combination of the two drugs has synergistic effect, which can exert stronger chemotherapy efficacy while reducing the side effects of chemotherapy drugs[11].

Quercetin, as an important component of phytoestrogens, is endowed with lipophilicity by its molecular structure, and the molecules can cross the cell membrane and enter cells to mediate various signal pathways related to the growth, apoptosis and metastasis of tumor cells. Quercetin can induce cell cycle arrest of tumor cells in the S phase, and affect DNA replication, so that the cells cannot enter next cell cycle, thus inhibiting tumor cell proliferation in a time-concentration dependent manner[12]. This inhibitory effect of quercetin is most sensitive to tumor cells in the proliferative phase, and the important way of its anticancer effect is to induce apoptosis[12-13]. Quercetin can also block the cell cycle in the G_1 phase, interfere with DNA replication, and down-regulate the level of Cyclin D1 related to cell cycle, thus prolonging G_1 phase[14].

The results showed that quercetin could inhibit the proliferation and induce apoptosis of MCF-7 cells in a concentration- and time-dependent manner, and the cell morphology and apoptosis morphology changed. After quercetin acted on MCF-7 cells for 24 h, a series of changes occurred in cell morphology. With the concentration of quercetin increasing, the number of MCF-7 cells gradually decreased, the cell contour became blurred, and the phenomena of volume shrinkage, rounding, shedding and even cytolytic changes occurred. The nuclear volume of apoptotic cells was reduced, and the chromatin was unevenly distributed, and a large number of apoptotic bodies could be seen. The apoptosis phenomenon was concentration-dependent at the same time. Meanwhile, the results showed that quercetin had little effect on the proliferation of normal breast epithelial cells, indicating that quercetin has little toxicity to normal cells and high safety. The scratch healing experiment showed that the healing ability of MCF-7 cells gradually decreased with the concentration of quercetin increasing, which indicated that quercetin could inhibit the migration ability of MCF-7 cells. To sum up, quercetin could inhibit the proliferation of breast cancer cells MCF-7, induce apoptosis and inhibit their migration ability, which can provide a new idea for improving the efficacy and tolerance of clinical chemotherapy drugs.

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