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Influence of luteolin on the apoptosis of esophageal cancer Eca109 cells and its mechanism of action

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ABSTRACT

The present study was conducted to verify the influence of luteolin on apoptosis of Eca109 cells and to further investigate the possible mechanisms underlying its effect on apoptosis. The cells were exposed to different concentrations of luteolin (0, 40, 80, 120, 160, 200, 240 μM) for 24, 48, and 72 h respectively. The influence of luteolin on proliferation of Eca109 cells was detected using MTT assay. Eca109 cells were then treated with luteolin (0, 40, 160, 240 μM) for 24 h. The effect of luteolin on cell cycle progression and apoptosis was assayed by using flow cytometry (FCM). Expression of caspase9 and caspase3 mRNA and protein was analyzed by real-time PCR and Western blot respectively. The results showed that luteolin could inhibit the proliferation of Eca109 cells at all concentrations in a time-dependent manner and the relative inhibition rate showed an inverted U-shaped association with the concentration of luteolin. Further, the cell cycle was arrested in the S phase following treatment with luteolin. Apoptosis analysis indicated that luteolin could induce the apoptosis of Eca109 cells across the three concentration groups, which exhibited a trend of first promotional and then inhibitory with the increases in luteolin concentration. The effect of luteolin on the mRNA and protein expression of caspase 9 and caspase3 first manifested as promotion, then inhibition. Therefore, luteolin may serve a role in promoting cell apoptosis by inducing Eca109 cell apoptosis that involves the expression of caspase3, caspase9 mRNA and protein. This study provides theoretical basis for further study and clinical application of luteolin. The specific mechanism has not yet been clarified and the other activation pathways inducing apoptosis need to be further studied.

1. Introduction

Esophageal cancer (EC) is the eighth most prevalent cancer worldwide, with an estimated 456,000 new cases in 2012 (3.2% of the total cancer cases), and the sixth leading cause of cancer-related mortality with an estimated 400,000 deaths (4.9% of the total cancer-related mortalities) [1]. The two major histological subtypes of EC are Esophageal squamous-cell carcinoma (ESCC) and esophageal adenocarcinoma [2]. Regarding ESCC, 79% of ESCC the total cases worldwide were diagnosed in Central and South-Eastern Asia [3]. EC is the third most common cancer and the fourth leading cause of cancer-related in China, with ESCC accounting for up to 90% of cases [4,5]. Although EC diagnosis and therapy have been improving, the overall 5-year survival rate of patients remained low at 15% to 20% [6]. In order to improve the survival rate of patients, novel anti-tumor agents have been investigated, with much attention on traditional Chinese medicines, due to their apparent numerous biological activities and low rate of side effects [7–9].

Flavonoid-based compounds are among the traditional Chinese medicines, and their anti-tumor effects have been widely studied. The main anti-tumor mechanisms of flavonoids may include anti-oxidative and anti-free radical effects, induction of tumor cell apoptosis, and influence on cell cycling [10]. Luteolin, an active flavonoid compound in natural resources, has been reported to exhibit various biological activities and therapeutic effects,

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including inhibiting the proliferation of cancer cells and inducing apoptosis [11–14]. A study demonstrated that luteolin has a good anti-tumor effect through various functions as a possible tumor suppressor including inhibition of cancer cell proliferation, induction of cancer cell apoptosis, autophagy, stagnation of the cell cycle, inhibition of tumor angiogenesis and reversal of tumor multidrug resistance [15]. Study of interference of luteolin exposure of human colon cancer cells and xenografts has suggested that luteolin may against human colon cancer as a potential chemopreventive and chemotherapeutic agent [16]. In addition, Tsai et al. [17] observed that luteolin could target cancer stem cells and reduce the invasiveness of cancer cells, and suggested its potential as an anti-angiogenesis and anti-transfer agent. Experiments have also shown that luteolin has certain pro-apoptotic effects on osteosarcoma and melanoma cells [14]. Several studies on luteolin observed that luteolin not only had a good anti-tumor effect, but also was generally non-toxic to normal cells, which indicate its potential clinical value for further study [11–13]. However, there are few studies on the potential protective mechanism of luteolin in EC. Our previous study determined that a cause of luteolin anti-tumor effect against of EC in vitro was through regulation of apoptosis, whereby the percentage of apoptotic cells increased with the concentration of luteolin [18]. The present study was conducted to verify the influence of luteolin on apoptosis of Eca109 cells by assessing the influence of increasing concentration via annexin V-FITC/PI staining and to investigate the possible mechanisms underlying its effect on apoptosis. This may provide a theoretical basis for further study and clinical application of luteolin.

2. Materials and methods

2.1. Cell line and cell culture

The human ESCC cell line Eca109 was obtained from Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China). RPMI 1640 medium (Jiangsu KeyGEN Biotechnology Co., Ltd, Nanjing, China) supplemented with 10% fetal bovine serum (CLARK Bioscience, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone Co., USA) was used for culture of the Eca109 cell line; the cells were grown in a 37 °C, 5% CO_2, saturated humidity incubator (MCO-15A; SANYO, Japan). The Eca109 cells were subcultured with 0.25% Trypsin–EDTA (HyClone) and observed by inverted phase contrast microscopy. The Eca109 cells at exponential phase were selected for subsequent experiments. Luteolin was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China).

2.2. MTT assay

Eca109 cells were plated at 1×10^4 cells/well in 96-well plates and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation for 24 h, when the cells were adhesive, various concentrations of luteolin (0, 40, 80, 120, 160, 200, 240 μM) were added to establish treatment groups; 37 μM doxorubicin (DOX, Harvey-bio Co., Ltd., Beijing, China) was added as a positive control group. Each exposure was performed in six wells, with incubation for 24, 48, and 72 h. Subsequently, 20 μL MTT (Nanjing Kaiji Biotech Development Co., Ltd., Nanjing, China) was added into each well and the cells were incubated for another 4 h. After removing the supernatants, 150 μL dimethylsulfoxide (Nanjing Boquan Co., Ltd., Nanjing, China) was added to each well and gently mixed on a plate oscillator for ~10 min to dissolve the formazan crystals. The OD_{570} was measured using a microplate reader (RT-6000; Rayto, USA) to quantify inhibitory effect. All experiments were performed three times. The equation to determine growth inhibition rate as follows: Growth inhibition rate (% of control) = (OD_{570} of control cells − OD_{570} of treated cells)/OD_{570} of control cells × 100%.

Table 1
Primers used for real-time PCR analysis.

Genes	Primer sequences	
GAPDH	F	GACAACAGCCTCAAGATCATCAG
	R	ATGGCATGGACTGTGGTCATGAG
Caspase9	F	CTAGTTTGCCCACACCCAGT
	R	TGCTCAAAGATGTCGTCCAG
Caspase3	F	GTGGAGGCCGACTTCTTGTA
	R	TGTCGGCATACTGTTTCAGC

2.3. Flow cytometry analysis of cell cycle progression

A total of 1×10^5 cells/well were seeded in 6-well plates and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. When the cells were in logarithmic growth phase, they were exposed to different concentrations of luteolin (0, 40, 160 or 240 μM) or 37 μM doxorubicin for 24 h. Then, the cells were trypsinized and cell density was adjusted to 1×10^6/mL with 1 mL PBS, which was followed by fixing with precooled 70% ethanol overnight at 4 °C. The cells were centrifuged at 300 × *g* for 5 min and washed with PBS, then incubated with 100 μL RNase at 37 °C for 30 min. Prior to analysis, 400 μL propidium iodide (PI) was incubated with cells in the dark for 30 min. Thereafter, cell cycle analysis was performed with a FACS420 with ModFit software.

2.4. Flow cytometric apoptosis assay

The effect of luteolin on apoptosis was determined with Annexin V-FITC/PI apoptosis detection kit (Jiangsu Keygen Biotech Co., Ltd, Nanjing, China). A total of 1×10^5 Eca109 cells/well were seeded into 6-well plates. When the cells were in logarithmic growth phase, the cells were exposed to different concentrations of luteolin (0, 40, 160, 240 μM) and 37 μM doxorubicin for 24 h. Then the cells were trypsinized and washed twice with PBS. A total of 5×10^5 cells were collected and suspended in 500 μL Binding Buffer, then mixed with 5 μL Annexin V-FITC and 5 μL PI. The cells were incubated at room temperature in the dark for 15 min, and then the rate of apoptosis in all groups was examined by flow cytometry (BD FACSCALIBUR; BD Bioscience, USA).

2.5. Realtime-PCR analysis

A total of 5×10^4 Eca109 cells per well were seeded into 6-well culture plates and cultured in a 37 °C, 5% CO_2, saturated humidity incubator for 24 h. The supernatant was discarded and the cells were treated with 37 μM doxorubicin or different concentrations of luteolin (0, 40, 160, 240 μM) for 24 h. The cells were then trypsinized and lysed. RNA was isolated using Trizol reagent according to the manufacturer's protocol, and a UV spectrophotometer was used to detect the purity and concentration of RNA. Quantified samples were used as template to synthesize cDNA with Reverse Transcription System (A3500; Promega Corporation, USA).

Eca109 cell cDNA was used as a template for amplification with targeted primers (Table 1). The reaction conditions of the PCR were heating at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The expression of GAPDH was used as internal reference.

2.6. Western blot analysis

The Eca109 cells were seeded in a 6-well plate at a density of 5×10^4/mL and cultured in a 37 °C, 5% CO_2 incubator for 24 h. The cells were treated with luteolin (0, 40, 160, 240 μM) or 37 μM

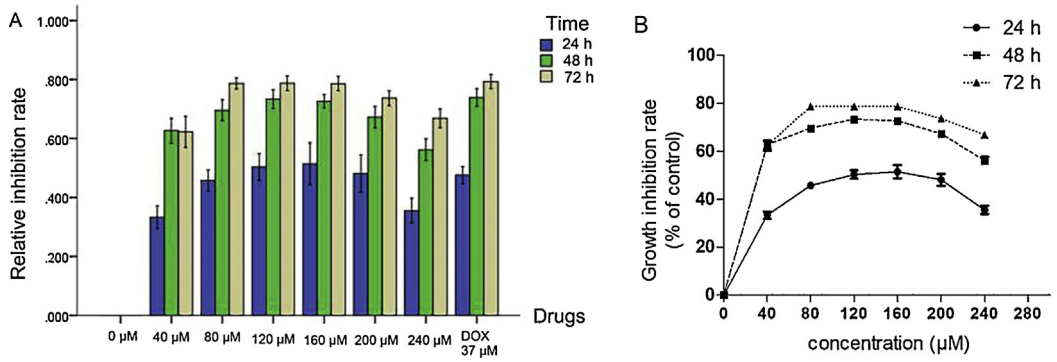

Fig. 1. Effect of drug treatment on the viability of Eca109 cells. (A) Drug treatment inhibited the growth of Eca109 cells. (B) Luteolin inhibited the growth of Eca109 cells over different time periods.

doxorubicin, and cultured under the same conditions for 24 h. The cells were then washed three times with cold PBS, and lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) with phenylmethyl sulfonylfluoride (Beyotime Institute of Biotechnology, Jiangsu, China). After 5 min, the cells were scraped and collected in a centrifuge tube over ice for 30 min, and finally centrifuged at 12,000 × *g* for 15 min at 4 °C. The protein concentration was determined with a bicinchoninic acid protein assay kit (Wuhan ServiceBio Technology Co., Ltd., Wuhan, China) according to the manufacturer's protocol. Proteins were separated by 8–12% SDS-PAGE (Wuhan ServiceBio Technology Co., Ltd., Wuhan, China). Proteins were fixed and stained with Bromophenol blue dye to determine the protein position and approximate concentration on blots ready for subsequent western blotting. The proteins were then transferred onto a polyvinylidene difluoride membrane (Wuhan ServiceBio Technology Co., Ltd.) membrane. Blocking was performed for 1 h at room temperature in tris-buffered saline containing 0.05% Tween-20 (TBST) and 5% w/v non-fat milk with continuous shaking. Subsequently, the membranes were respectively incubated with primary antibody against Caspase 3 (1:1000, ab90437, Abcam, Cambridge, UK), Caspase 9 (1:1000, 10380-1-AP, PTG, China). The membranes were then washed 3 times with TBST and incubated with HRP-conjugated secondary antibody (1:3000) at room temperature for 30 min. Detection was achieved by electrochemiluminescent reaction (Bio-Rad), according to the manufacturer's instructions and autoradiography on X-ray film. Target protein expression was normalized to that of β-actin.

2.7. Statistical analysis

The results are reported as the mean ± standard deviation. Dunnet's-*t* tests were used to analyze the differences between the control and treated samples in SPSS 19.0 statistic software (IBM, Corp., Armonk, NY, USA). A difference at $P < 0.05$ was considered statistically significant.

3. Results

3.1. Influence of luteolin on the proliferation of Eca109 cells

Treatment of EC Eca109 cells with different concentrations of luteolin could inhibit cell proliferation (Fig. 1A). Following treatment for 24, 48 and 72 h, the rate of cell proliferation inhibition in each treatment group was significantly higher than that of negative control group ($P < 0.05$). With the increase of luteolin concentration, the inhibitory effect on cancer cells first increased and then decreased, and relative inhibition rate exhibited an inverted U-shaped association with luteolin concentration (Fig. 1B). After treatment for 24 h, the highest inhibition rate was 51.4 ± 6.8% at the concentration of 160 μM; after treated for 48 h, the highest inhibition rate was 73.4 ± 3.0% at the concentration of 120 μM. However, the inhibitory effect on cell proliferation did not differ significantly at a concentration of 40 μM luteolin between cells treated for 48 and 72 h. The highest inhibition rate was 78.7 ± 2.4% at a concentration of 120 μM after treatment for 72 h (Table 2). 40, 160 and 240 μM (higher than those tested in previous study by our group) were selected as experimental concentrations for further experiments.

Table 2
The inhibition rate of Eca109 cells after treatment with different concentrations of luteolin ($n = 6$, $\bar{x} \pm s$ %).

Groups	Concentration (μM)	24 h	48 h	72 h
Luteolin	0	–	–	–
	40	33.3 ± 3.6*,#	62.7 ± 4.0*,#	62.3 ± 5.1*,#
	80	45.8 ± 3.4*	69.6 ± 3.4*	78.7 ± 1.8*
	120	50.3 ± 4.3*	73.4 ± 3.0*	78.7 ± 2.4*
	160	51.4 ± 6.8*	72.6 ± 2.1*	78.6 ± 2.3*
	200	48.1 ± 6.1*	67.3 ± 3.5*	73.7 ± 2.4*
	240	35.5 ± 4.0*,#	56.2 ± 3.5*,#	66.9 ± 3.0*,#
DOX	37	47.6 ± 2.7*	74.9 ± 2.8*	79.3 ± 2.3*

* Two-sided Dunnett-*t* test, $P < 0.05$ compared with blank control group.
\# Two-sided Dunnett-*t* test, $P < 0.05$ compared with positive control group.

3.2. Effect of luteolin on the cell cycle of EC Eca109 cells

The data in Fig. 2 demonstrate that the cell cycle was altered by luteolin. The effect of luteolin on the cell cycle of Eca109 cells mainly impacted on the S and G2/M phases. When luteolin was applied at the concentration of 160 or 240 μM, the percentage of cells in S phase was significantly higher than in the negative control group, while the percentage of cells in G2/M phase was significantly lower than in the negative control group (Fig. 2B, $P < 0.05$), suggesting that the cells were arrested at the S phase.

3.3. Effect of luteolin on the apoptosis of EC Eca109 cells

Following the treatment of Eca109 cells treated with 0, 40, 160, 240 μM luteolin, or 37 μM doxorubicin for 24 h, flow cytometry was used to detect the rate of apoptosis in each group. The cell maps are displayed in Fig. 3. The results demonstrated that the rate of apoptosis did not differ between the 40 μM luteolin-treated group and the negative control group ($P > 0.05$). The effect of 160 or 240 μM luteolin on the apoptosis of Eca109 cells was significant compared with negative control ($P < 0.05$), and comparable to that of 37 μM doxorubicin ($P > 0.05$). The effect of luteolin on the apoptosis of Eca109 cells across the three concentration groups exhibited a trend of first promotional and then inhibitory with the increases in luteolin concentration, suggesting that luteolin had an association with

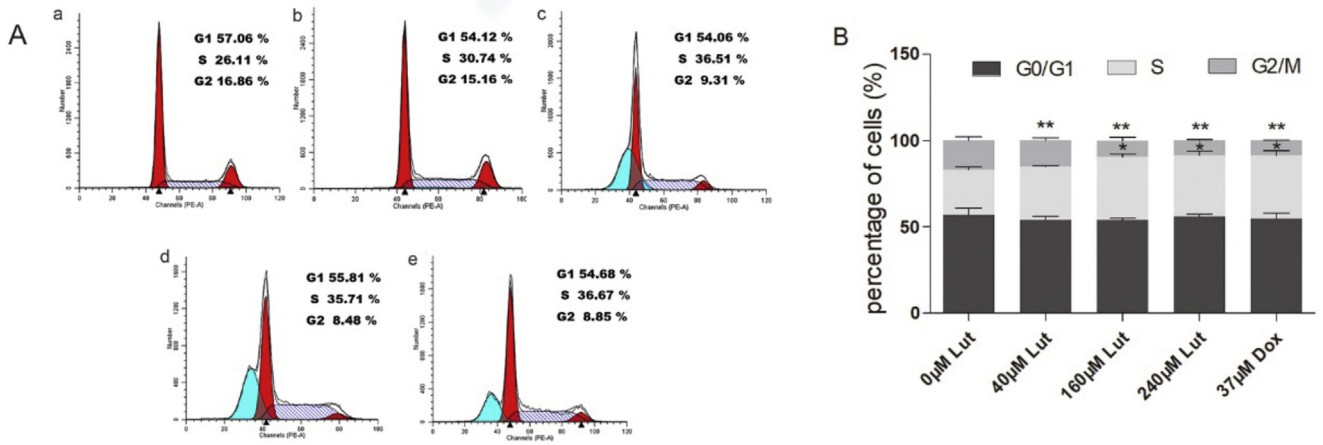

Fig. 2. (A) Effect of luteolin on the cell cycle of Eca109 cells. (a) Negative control group; (b) 40 μM luteolin; (c) 160 μM luteolin; (d) 240 μM luteolin; (e) 37 μM DOX. DOX, doxorubicin. (B) Percentages of Eca109 cells in cell cycle phases. *The percentage of cells in S phase $P < 0.05$ vs negative control group **The percentage of cells in G2/M phase $P < 0.05$ vs negative control group.

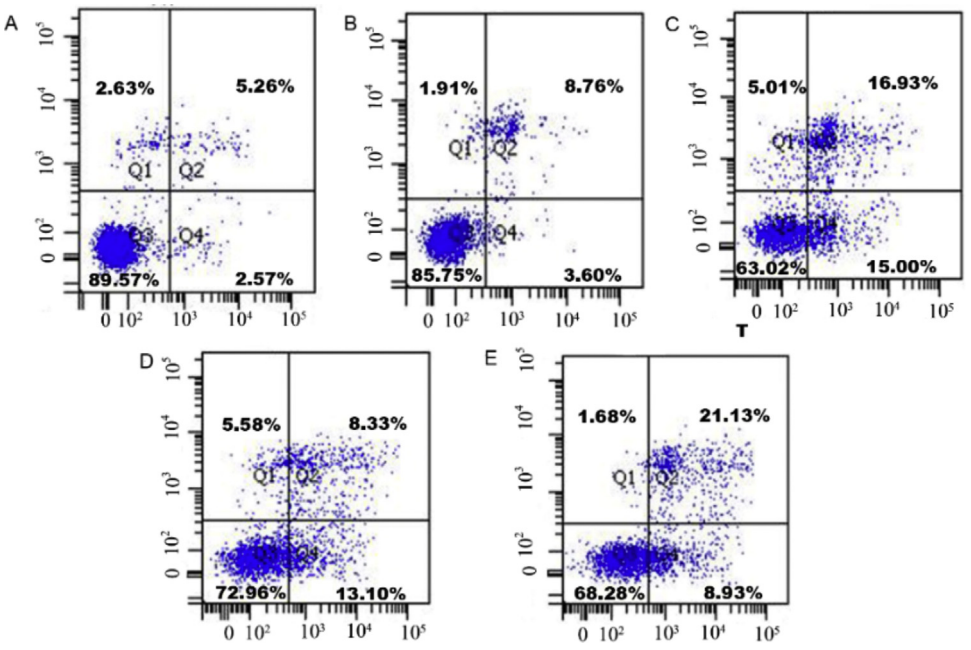

Fig. 3. Effect of different concentrations of luteolin on the apoptosis of Eca109 cells. (A) Negative control group; (B) 40 μM luteolin; (C) 160 μM luteolin; (D) 240 μM luteolin; (E) 37 μM DOX. DOX, doxorubicin. In each cell map, the lower right quadrant represents early apoptotic cells and the upper right quadrant represents late apoptotic or necrotic cells.

relationship on the apoptosis of Eca109 cells. Statistical analysis of the proportion of early apoptotic cells in each group suggested that the highest percentage of premature apoptotic cells occurred in the 160 μM group, which was with significance when compared with that in the negative control group ($P < 0.05$). These data suggested that luteolin can promote the apoptosis of EC Eca109 cells.

3.4. Effect of luteolin on the mRNA expression of caspase 9 and caspase 3 in Eca109 cells

Results of mRNA expression of caspase 9 and caspase 3 in the presence of different concentrations of luteolin are shown in Fig. 4. The mRNA expression of caspase 9 and caspase 3 in Eca109 cells was increased with 40 μM luteolin, but the mRNA expression of caspase 9 and caspase 3 were inhibited when the concentration of the luteolin increased to 160 μM and 240 μM (Fig. 4). The inhibitory effect of 160, 240 μM luteolin on the mRNA expression of caspase9 and caspase3 was not statistically different ($P > 0.05$). This suggested that the effect of luteolin on the expression of caspase9 and caspase3 mRNA appeared to be promoted and then inhibited with the increase in luteolin concentration.

3.5. Effect of luteolin on the protein expression of caspase 9 and caspase 3 in Eca109 cells

The effect of different concentrations of luteolin on the protein expression of caspase9 and caspase3 in Eca109 cells are shown in Fig. 5. The effect of luteolin on the protein expression of caspase9 and caspase3 was first promotional, then inhibitory. There was no significant difference in the expression level of caspase9 between 40 μM and 160 μM luteolin (Table 3, $P > 0.05$), while the expression of caspase9 and caspase3 on treatment with the 240 μM luteolin was inhibited significantly ($P < 0.05$).

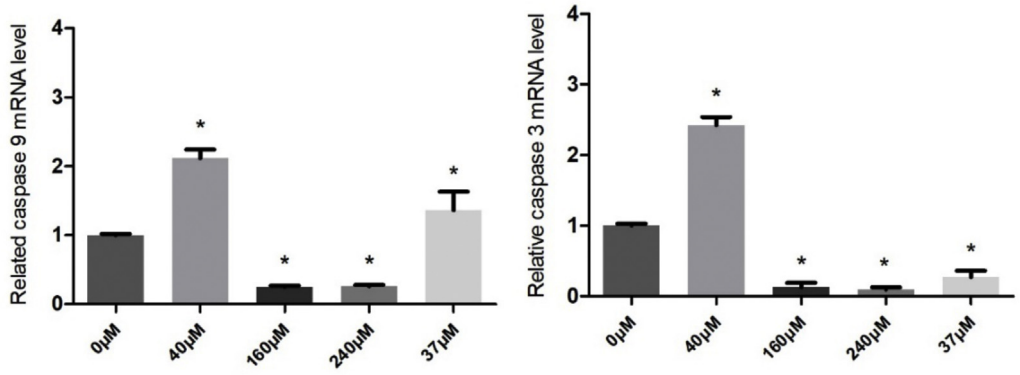

Fig. 4. Effect of luteolin on the mRNA expression of caspase 9 (A) and caspase 3 (B). *Two sided Dunnett-t test, $P < 0.05$ vs negative control group.

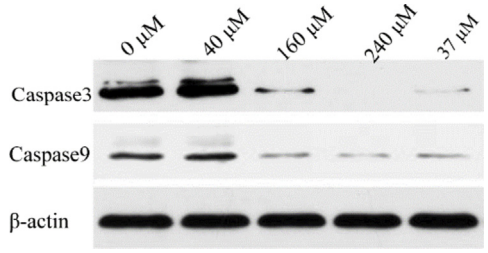

Fig. 5. Effect of luteolin on the protein expression of caspase 3 and caspase 9 protein. The 37 μM DOX group is also shown. DOX, doxorubicin.

Table 3
Effects of luteolin on expression of caspase9 and caspase3 protein ($n = 3, \bar{x} \pm s$).

Groups	Concentration (μM)	Genes	
		Caspase9	Caspase3
Luteolin	0	1.91 ± 0.11	0.77 ± 0.03
	40	2.35 ± 0.26	0.87 ± 0.03*
	160	1.40 ± 0.36	0.18 ± 0.03*
	240	0.86 ± 0.20*	0.02 ± 0.02*
DOX	37	1.14 ± 0.21	0.06 ± 0.02*

* Two-sided Dunnett-*t* test, $P < 0.05$ versus negative control group.

4. Discussion

The present study aimed to investigate the mechanism of action of luteolin in influencing the apoptosis of the EC cell line Eca109. Doxorubicin was selected as a positive control, since it is a broad-spectrum anti-cancer agent that is used for the clinical therapy of several cancers including prostate cancer, breast cancer, lung cancer and bladder cancer [19–22], limited in application by its side-effects such as cardiotoxicity [23,24]. Therefore, natural resources such as luteolin appear particularly important on account of their relatively low toxicity as well as potential anti-cancer activity. Studies have indicated that luteolin is capable of inhibiting the proliferation and inducing the apoptosis of many types of cancer cells in vitro, including gastric cancer, melanoma, prostate cancer, hepatocellular carcinoma, oral squamous cancer, lung cancer, cholangiocarcinoma and breast cancer cells [25–32].

Inhibition of tumor cell growth is among the main strategies employed for the treatment of tumors at present. In the current study, it was identified that luteolin could inhibit the proliferation of Eca109 cells at all concentrations in a time-dependent manner, which is consistent with the results of our previous study and of Zhang et al. [18,33]. With the increase in luteolin concentration, the inhibitory effect on Eca109 cells was first increased and then decreased, and relative inhibition rate exhibited an inverted U-shaped association with luteolin concentration, which differ from the reports of several authors that cytotoxicity in ESCC in a dose-dependent manner [18,33]. However, it was consistent with the results of Li et al. [34] and Atif et al. [35], suggesting a relationship based on a U-shaped concentration-response curve in certain instances, indicating a "hormesis" phenomenon [36].

Inhibition of cell growth is typically accompanied by redistribution of the cell cycle, as described by several authors [16,37,38]. In the current study, the percentage of cells in the G2/M phase increased while a decrease in S-phase cells was observed following treatment with luteolin; whereas the percentages of G0/G1 phase cells remained at almost the same levels in the negative control group. This was similar with findings on the effect of luteolin on the cell cycle of human colon cancer cells and human esophageal carcinoma cells treated with three different flavones in other studies [16,39]. This cell cycle arrest demonstrated the underlying action of luteolin in causing decrease in cell viability and induction of cell death.

The effect of luteolin on the apoptosis of Eca109 cells was detected by the Annexin V-FITC/PI method. It was observed that luteolin could induce apoptosis of Eca109 cells; in fact, the apoptotic rate first increased and then decreased with the increase of luteolin concentration. The maximum apoptotic rate was induced by 160 μM. There are two important pathways of apoptosis in mammals: the mitochondrial pathway and the extrinsic pathway. The former is the main target in the developing development of anti-tumor drugs [40–42]. Caspase3 is among the important proteins that participate in cell apoptosis mediated by the mitochondrial pathway, which is cleaved and activated by the initiator caspase9 [43].

According to real-time-PCR and Western blot analysis, it was indicated that the alterations in the expression of caspase9 and caspase3 at the mRNA level ultimately result in the alterations at the protein level. The maximum regulation of the mRNA and protein levels by luteolin was apparent in the 40 μM group, which differ to the results of flow cytometry. Ding et al. and Ma et al. [44,45] observed that 50 and 80 μM luteolin could activate caspase 9, leading to activation of caspase 3 and finally the promotion of apoptosis of hepatoma cells and lung cancer cells, which was consistent with the results of the current study. However, the intervention concentrations of luteolin in their studies were all within 100 μM. It was difficult to confirm the regulation of luteolin on Bax, Bcl-2, caspase9, caspase3, etc. with the increase of the concentration. The mechanism of action of luteolin in cancer cells is multi-targeted and interlinked. Caspase cascade is a common pathway of apoptotic pathways, and luteolin may be involved in other activation pathways that induce apoptosis.

In conclusion, it was suggested in the present study that luteolin may serve a role in promoting Eca109 cell apoptosis via a mechanism involving regulation of the expression of caspase 3 and

caspase 9 at the mRNA and protein levels. The specific mechanism is yet to be clarified and the other activation pathways inducing apoptosis should be studied further. The limitation of this study is that the effect and mechanism of luteolin were studied on only one human EC cell line. In future study, multiple cell lines will be tested to elucidate the general effect of luteolin on EC.

Conflict of interest

The authors confirm that the contents of this article have no conflict of interest.

Acknowledgments

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