Luteolin induces mitochondrial apoptosis in HT29 cells by inhibiting the Nrf2/ARE signaling pathway

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Abstract. The aim of the current study was to investigate luteolin-induced apoptosis and the molecular mechanisms underlying it in HT29 cells. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to assess the cytotoxicity of luteolin on HT29 cells, and a dichloro-dihydro-fluorescein diacetate assay was used to measure cellular levels of reactive oxygen species (ROS). The effects of luteolin on the mitochondrial membrane potential were also evaluated. Bax and Bcl-2 mRNA expression were determined using reverse transcription-quantitative PCR. Additionally, western blot analysis was performed to assess changes in cytochrome c and caspase-3 protein expression. Localization of nuclear factor erythroid 2-related factor 2 (Nrf2) in the nucleus was also assessed using immunofluorescence. Luteolin exhibited cytotoxicity on HT29 cells in a time- and concentration-dependent manner. Additionally, ROS production was indicated to be increased and ROS scavenging was decreased, which resulted in a significant increase in the levels of ROS in the cells. The mitochondrial membrane potential was indicated to decrease following luteolin treatment. At the molecular level, luteolin significantly increased the mRNA expression of Bax and the protein expression of cytochrome c, caspase-3, p47\textsuperscript{phox} and p22\textsuperscript{phox}. The results revealed that luteolin decreased Bcl-2 protein expression and inhibited the nuclear localization of Nrf2. In conclusion, the current study indicated that luteolin inhibited HT29 cell proliferation and induced apoptosis via the mitochondrial pathway.

Introduction

Colon cancer is a common malignant tumor of the digestive tract that can improve with lifestyle and diet modifications (1). Previously, there has been a decline in the incidence of CRC in adults older than 50 years in the United States; however, the number of new cases is expected to increase among young adults aged 20-49 years by 2030 (2). The 5-year survival rate for colon cancer is relatively high, however, the high recurrence rate and metastasis of the tumor to distant organs is the primary cause of mortality in patients with this disease (3,4). In recent years, colon cancer has become one of the main causes of cancer-associated mortality (5). Treatments used for colon cancer include surgery, radio- and chemotherapy. With the development of technology, these traditional methods have undergone a number of improvements (6,7). However, these improvements are insufficient in meeting clinical needs, including prolonging survival, preventing cancer metastasis, and reducing recurrence rates (4). To achieve an increased survival rate and improved treatment efficacy, the development and evaluation of phytochemicals that exhibit anticancer properties is urgently required.

Luteolin (3, 4’, 5, 7-tetrahydroxy-flavone) is an important flavonoid that can be found in honeysuckle, chrysanthemum, nepeta and Prunella vulgaris (8). Celery, sweet pepper, Chinese cabbage, cauliflower and Camellia sinensis also contain large quantities of luteolin (9). Luteolin has been revealed to exhibit anti-inflammatory, antioxidative and anticancer properties (9). Luteolin has also been reported to decrease serum glucose and exhibit a number of other pharmacological activities (8,10,11). Studies have demonstrated that luteolin can provide resistance against oncogenic stimulation in vivo and in vitro, inhibit cell proliferation, and induce cell cycle arrest and apoptosis by stimulating or inhibiting intracellular and extracellular signaling pathways (12,13). Furthermore, the efficacy of luteolin in treating colon cancer has been previously reported (14,15). Luteolin has been indicated to induce apoptosis in colon cancer cells by arresting the cell cycle at the G2/M phase (11,13). Recent research has revealed that the molecular mechanisms underlying the luteolin-induced apoptosis of colon cancer cells is associated with the inhibition of the Wnt/\beta-catenin/glycogen synthase kinase-3\beta (16) and phosphatidylinositol 3-kinase/Akt signaling pathways (17), reduction of antioxidant capacity (16) and the induction of changes in the ceramide/spHINGosine-1-phosphate ratio (18).

A variety of drugs exert an anticancer effect by increasing the level of reactive oxygen species (ROS) and activating the

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mitochondrial apoptosis pathway (19,20). It has been indicated that the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant responsive element (ARE) signaling pathway is an important pathway during the cellular antioxidant response (21). Furthermore, it has been demonstrated that the regulation of antioxidant enzymes and phase II detoxification enzymes via this signaling pathway can result in the scavenging of ROS and other harmful substances (22).

The current study was performed to investigate whether luteolin induces mitochondrial apoptosis in the colon cancer cell line HT29 by inhibiting the Nrf2/ARE signaling pathway.

Materials and methods

Cells and reagents. HT29 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences.

Luteolin was purchased from Dalian Meilun Biology Technology Co., Ltd. MTT (cat. no. 298-93-1), DMSO (cat. no. 68-67-5), FBS and high-glucose DMEM were purchased from Beijing Solarbio Science & Technology Co., Ltd. Dichloro-dihydro-fluorescein diacetate (DCFH-DA, cat. no. d6883) was obtained from Sigma-Aldrich; Merck KGaA. RIPA buffer (cat. no. P0013C), SDS-PAGE gel preparation kit (cat. no. P0012A) and Mitochondrial Membrane Potential Detection kit (cat. no. C2006) were purchased from Beyotime Institute of Biotechnology. RNAiso Plus (cat. no. 9108; Takara Biotechnology Co., Ltd.), the PrimeScript RT Reagent kit (cat. no. RR047a) and TB Green Premix Ex Taq II kit (cat. no. RR820) were obtained from Takara Bio, Inc., rabbit anti-cytochrome c (cyt C) monoclonal antibody (1:2,500; cat. no. ab133504), rabbit anti-caspase-3 monoclonal antibody (1:500; cat. no. ab197202), rabbit anti-p47phox monoclonal antibody (1:2,500; cat. no. ab181090), rabbit anti-p22phox monoclonal antibody (1:2,000; cat. no. ab191512), rabbit β-actin antibody (1:1,000; cat. no. bs-0061R), goat anti-rabbit IgG-HP (H+L) secondary antibody (1:1,000; cat. no. E030120), rabbit anti-Nrf2 monoclonal antibody (1:250; cat. no. ab62352) and Alexa Fluor® 647-labeled goat anti-rabbit fluorescent secondary antibody (1:500; cat. no. ab150079) was purchased from Abcam. The primers used in the RT-qPCR were as follows: Bax forward, 5'-CTTCCGGAGGAGACTAGTGA-3' and reverse, 5'-CTTCTCCGTGCTCCATC-3' (NG_012191; length 318); Bcl-2 forward, 5'-AGGATTGTGCGCTTCTTTGAGT-3' and reverse, 5'-ACTGGTACATGGAACCTTTTGCAT-3' (NG_009936; length 335) and β-actin forward, 5'-CGCGAGAAGACCCAGAT-3' and reverse, 5'-GCCGTTGTTTGCCGTACAGG-3' (NG_007992; length 550).

**MTT assay.** Cells in the log growth phase were digested to obtain a single-cell suspension with a density of 1.5x10⁵ cells/ml. Subsequently, 100 µl cell suspension cultured in high-glucose DMEM containing 10% FBS was added to each well of a 96-well plate, after which the plate was incubated for 24 h (37°C; 5% CO₂). Luteolin was added to the wells at a final concentration of 10, 20, 40, 80 or 160 µM, with six parallel wells for each group. 100 µl of solvent (1% DMSO) was added to the blank control cells. Cells were treated for 24, 48 or 72 h. A total of 20 µl MTT solution (5 mg/ml) was subsequently added to each well, after which the plate was incubated at 37°C for an additional 4 h. The supernatant was discarded and the MTT in each well was dissolved in 150 µl DMSO. Optical density (OD) was measured at 490 nm and inhibition rate (IR) was calculated as follows: IR(%)=[OD(blank control)-OD(experimental group)/OD(blank control)] x blank. The half-maximal inhibitory concentration (IC₅₀) values of luteolin on HT29 cells were determined by a plotting dose-response curve.

**Measurement of intracellular ROS level.** A cell suspension containing 5x10⁵ cells/ml was prepared from HT29 cells in the log growth phase. A total of 5x10⁵ cells/well were inoculated into a 6-well plate and cultured overnight. After the cells adhered to the plate, 20 or 40 µM luteolin was added, and an equal volume of DMSO was added to the blank control cells. After 48 h, the culture medium (high-glucose DMEM containing 10% FBS) was aspirated. A total of 10 µmol/l DCFH-DA serum-free medium was then added to the culture. The mixture was lightly agitated until all the cells were covered, followed by incubation at 37°C for 20 min. Cells were then rapidly washed three times with serum-free medium to remove DCFH-DA that did not enter the cells. Finally, a total of 1x10⁵ cells/field of view were imaged using a fluorescence microscope under x20 magnification. The fluorescence intensity was measured (Image Pro Plus 6.0; Media Cybernetics) to determine the level of intracellular ROS.

**Immunofluorescence.** HT29 cells in the log growth phase were digested with trypsin to obtain a single-cell suspension containing 1x10⁶ cells/ml. A total of 2 ml suspension was subsequently placed in each well of a six-well plate with coverslips at the bottom of each well. After adhesion, cells were washed once with PBS. A total of 1 ml culture medium (high-glucose DMEM containing 10% FBS) and 1 ml JC-1 staining solution were placed in each well of a six-well plate with coverslips at the bottom of each well. After adhesion, cells were treated as aforementioned. After 48 h of treatment with luteolin, the culture medium was aspirated and the cells were washed once with PBS. A total of 1 ml culture medium (high-glucose DMEM containing 10% FBS) and 1 ml JC-1 staining solution were successively added to the suspension, which was then mixed thoroughly. Cells were incubated at 37°C for 20 min and digested using trypsin. A multifunctional spectrophotometer was used to measure absorbance at excitation and emission wavelengths of 525 and 590 nm, respectively.

**RT-qPCR.** HT29 cells in the log growth phase were treated with 48 h with 20 or 40 µM luteolin. Cells were subsequently fixed with 4% paraformaldehyde at 37°C for 30 min, permeabilized with 0.3% Triton X-100 for 15 min at room temperature, blocked in PBS containing 1% BSA for 1 h at 37°C, incubated with primary antibodies against Nrf2 (1:250) overnight at 4°C, washed, treated with Alexa Fluor® 647-labeled goat anti-rabbit fluorescent secondary antibody (1:500) for 30 min at 37°C, washed and imaged using a fluorescence microscope under x200 magnification.
The supernatant was collected and 400 µl isopropyl alcohol was added. The mixture was left to stand and subsequently centrifuged at 13,000 x g for 10 min at 4°C. The supernatant was discarded and the precipitate obtained was washed with 75% ethanol. The mixture was then centrifuged to remove the ethanol and the supernatant was discarded. The precipitate was dried using super-clean bench at room temperature for 2 min, and dissolved in 20 µl nuclease-free water at room temperature. Genomic DNA removal and RT were performed using a reaction kit with the following thermocycling conditions: 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. During qPCR, denaturation was performed at 95°C for 10 min and amplification was performed at 95°C for 15 sec, 60°C for 15 sec and 72°C for 30 sec for 40 cycles. Data were analyzed using the 2^{−ΔΔCq} method (23) and normalized to the internal reference gene β-actin.

**Western blot analysis.** HT29 cells in the log growth phase were treated for 48 h with 20 or 40 µm luteolin or with an equal volume of vehicle that was used as the blank control. RIPA buffer was added to the cells on ice and cells were ground. The lysate was kept at 4°C for 30 min and subsequently centrifuged at 13,000 x g for 10 min at 4°C. Supernatant was collected and a BCA assay kit was used to measure protein concentration. Afterwards, 4X loading buffer was added the remaining supernatant, and the 30 µg of protein sample was denatured and added to a 4% stacking/10% resolving gel. Samples were then blotted onto a PVDF membrane, blocked with 5% skim milk in TBS containing 0.05% Tween-20 (TBS-T) for 1 h at room temperature, incubated with primary antibodies against cyt C (1:2,500), caspase-3 (1:500), p47\textsuperscript{phox} (1:2,500), p22\textsuperscript{phox} (1:2,000) and β-actin (1:1,000) overnight at 4°C and incubated with secondary antibodies goat anti-rabbit IgG-HRP (H+L) (1:2,000) and anti-mouse IgG-HRP (H+L) (1:1,000) for 1 h at room temperature. Enhanced chemiluminescence, X-ray development, gel imaging (Bio-Rad ChemiDoc XRS+ 170-8625; Bio-Rad Laboratories, Inc.) and image analysis (ImageJ 1.8; National Institutes of Health) were subsequently performed.

**Statistical analysis.** SPSS (version 21.0; IBM Corp.) was used for data analysis. One-way ANOVA was used for comparison among multiple groups with LSD post-hoc tests. P<0.05 was considered to indicate a statistically significant result. Data are expressed as the mean ± SEM.

**Results**

The cytotoxicity of luteolin on HT29 cells. Fig. 1 indicated that for each exposure period, as the concentration of luteolin was increased, the cytotoxicity of luteolin on HT29 cell gradually increased. The half-maximal inhibitory concentration (IC\textsubscript{50}) values of luteolin were calculated as 69.66±3.42, 28.94±2.37 and 22.30±3.05 µM when cells were treated for 24, 48 and 72 h, respectively. Furthermore, for each concentration, the cytotoxicity of luteolin gradually increased for the different incubation periods.

Effect of luteolin in HT29 cells. Fig. 2 demonstrated that as the luteolin concentration was increased, the ROS level in the HT29 cells increased.

Fig. 3 indicated that as the luteolin concentration was increased, the mitochondrial membrane potential of HT29 cells decreased. The mitochondrial membrane potential was indicated to be significantly lower in cells treated with 20 and 40 µM luteolin compared with blank control cells.

Fig. 4 demonstrated that as the luteolin concentration was increased, mRNA expression of Bax and of Bcl-2 decreased. These results indicated that Bcl-2 can prevent multiple signaling pathways from blocking cell apoptosis and prolonging cell survival. Bax is a mitochondrial membrane protein that can promote or mediate apoptosis (24,25). Additionally, the mRNA expression of Bax and Bcl-2 were significantly different in the cells treated with 20 and 40 µM compared with the respective blank control cells.

The results indicated that as the luteolin concentration was increased, the protein expression of cyt C and caspase-3 in the HT29 cells gradually increased (Fig. 5). Additionally, p47\textsuperscript{phox} and p22\textsuperscript{phox} protein levels in the cells gradually increased (Fig. 6).

Fig. 7A and B demonstrated that as the luteolin concentration increased, nuclear Nrf2 localization in HT29 cells decreased.

**Discussion**

Dysregulated cell proliferation is a characteristic of a large number of cancer types (26). Therefore, the induction of apoptosis is a beneficial characteristic for anticancer drugs to exhibit. Previous studies have demonstrated that resveratrol, matrine, quercetin and a number of other phytochemicals induce apoptosis and inhibit the proliferation of liver, stomach, oral, skin and colon cancers (27-29). Luteolin is a flavonoid that has been indicated to arrest the cell cycle and induce apoptosis in a variety of different cancer types (13,14,27). In the present study, the results of the MTT assay demonstrated that luteolin exhibited cytotoxicity on HT29 cell in a concentration- and time-dependent manner, which is consistent with the results of other studies (17,30).

Signaling networks, including pathways associated with cell proliferation, are modified in a number of cancer types, which makes cells unable to regulate their properties (31). Dysregulated proliferation is the basis of the development and
progression of cancer, and it has been revealed that apoptosis and cell cycle signaling pathways, and proteins associated with these processes, are directly associated with cancer cell proliferation (32,33). Apoptosis may occur via the mitochondrial or death receptor pathway (34). The majority of tumors avoid intrinsic and extrinsic apoptosis by modifying the expression or structure of proteins associated with apoptosis, which causes cancer cells to exhibit higher error tolerance and resistance (33). Therefore, the induction of apoptosis is an important anticancer mechanism that can be identified in chemotherapeutic agents.

During the initiation of apoptosis, an abnormal increase in ROS occurs, which is an important stimulus for mitochondrial apoptosis (35). It has been demonstrated that a number of drugs induce apoptosis of cancer cells by increasing ROS levels (36,37). In the present study, it was indicated that luteolin induced an increase in ROS level in HT29 cells. Changes in cellular ROS levels are directly associated with changes in mitochondrial function, with abnormalities in the electron permeability of the mitochondrial membrane leading to significant changes in cellular ROS levels (38). Additionally, mitochondria are most vulnerable to ROS attack (39,40). In the present study, luteolin decreased the mitochondrial membrane potential in HT29 cells. Therefore, the luteolin-induced increase in mitochondrial membrane permeability may result in increased ROS levels in HT29 cells. However, the current study did not use normal human colon cells as a control, which may lead to the inability of the present study to directly determine whether luteolin induces increased ROS in normal colon cells, which would allow for the specificity and side effects of luteolin to be assessed accurately. Therefore, future studies will aim to determine the effect of luteolin on the levels of ROS and apoptosis in normal human colon cells.

Changes in mitochondrial membrane permeability are determined by the relative levels of Bcl-2 and Bax. In the mitochondrial apoptotic pathway, Bax promotes apoptosis by damaging mitochondrial membrane integrity, whereas Bcl-2 inhibits apoptosis by maintaining the integrity of the mitochondrial membrane (41). The results of the present study indicated that luteolin increased Bax mRNA expression and downregulated Bcl-2 mRNA expression.
As mitochondrial membrane permeability changes, the movement of cyt C from the mitochondria to the cytoplasm is important in the initiation of the caspase apoptosis cascade (42). The results of the current study indicated that luteolin increased the expression of cyt C and caspase-3 proteins in HT29 cells, and the increase of cyt C and caspase-3 protein levels make HT29 cells more sensitive to the intrinsic apoptotic pathway (43). The results of the current study revealed that luteolin induced apoptosis of HT29 cells by stimulating the mitochondrial apoptotic pathway. However, the present study only demonstrated that luteolin induced mitochondrial apoptosis in HT29 cells through the detection of mitochondrial permeability and mitochondrial apoptosis-associated proteins and did not identify the modification of mitochondria. The modification of mitochondria can directly reflect the occurrence of mitochondrial apoptosis (44). Therefore, future studies may assess the modification of mitochondria.
Luteolin can increase ROS levels in HT29 cells. Increased endogenous cellular ROS levels are associated with ROS production and clearance pathways (45). NADPH oxidase (NOX) is a membrane protein that is widely distributed in tissues and organs (46). NOX reduces oxygen molecules in the body to superoxide anions through NADPH-dependent electron transfer (47). Furthermore, NOX has been indicated to be responsible for ROS generation in the body (48). NOX exhibits little catalytic activity and binds to a number of regulatory subunits to form a stable complex before it can exert any catalytic activity (49), including p47phox and p22phox, which are important cofactors that are required for the stability of NOX. In the present study, luteolin was revealed to promote p47phox and p22phox expression in HT29 cells. This result suggested that luteolin may increase ROS levels in HT29 cells by increasing NOX stability. Furthermore, cellular ROS levels determine the activity of the antioxidant system (50). Recent studies have demonstrated that the Nrf2/ARE signaling pathway is an essential pathway during antioxidant response in cells (51). The regulation of antioxidant enzymes and phase II detoxification enzymes by this signaling pathway can result in the ROS scavenging, which can result in a detoxifying and neutralizing effect (22).

The results of the present study also indicated that luteolin inhibited Nrf2 activation, blocked nuclear localization of Nrf2 and inhibited the expression of antioxidant enzymes. Therefore, it was revealed that luteolin prevented Nrf2 activation and promoted abnormal ROS level increases in HT29 cells by modulating the expression of p47phox and p22phox. ROS levels in cancer cells are high compared with normal cells. Furthermore, in cancer cells that are adapted to high ROS levels, further increases in ROS levels can promote cell apoptosis.

However, other studies have demonstrated that luteolin can induce the demethylation of the Nrf2 gene promoter region, upregulate the Nrf2 gene expression, activate the Nrf2/ARE pathway, increase the antioxidant capacity, inhibit the transformation and promote apoptosis of colon cancer cells (52,53), which is not supported by the results of the present study. In the current study, it was speculated that the antioxidant capacity served a different role at different stages of cancer development. The increase of antioxidant capacity reduces the level of ROS, protects cells from DNA damage caused by oxidative stress, and inhibits further transformation of tumors. Conversely, the improvement of antioxidant capacity exhibits an increase drug resistance and oxidation resistance ability to tumor cells, promoting the development of tumors.

In conclusion, luteolin induced apoptosis in HT29 cells by promoting ROS production and inhibiting ROS scavenging through stimulating the mitochondrial apoptotic pathway. However, the current study did not identify a useful luteolin inhibitor, and the effects of luteolin on the proliferation, apoptosis and ROS production of HT29 cells could not be fully identified. Future studies will aim to identify a suitable luteolin inhibitor for use in subsequent research.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
FJX and NC designed the research. FJX performed the MTT assay, measurement of intracellular ROS levels and measurement of mitochondrial membrane potential. WLY performed immunofluorescence experiments, RT-qPCR, and western blot analysis. HY and BFL analyzed and interpreted the data and finalized the manuscript. All authors read and approved the final version of the manuscript.

Figure 7. Immunofluorescence analysis of Nrf2 localization in HT29 cells treated with luteolin. (A) Expression of Nrf2 was determined by immunohistochemistry staining. (B) Fluorescence intensity of Nrf2. Data are expressed as the mean ± SEM. *P<0.05 vs. control. Nrf2, nuclear factor erythroid 2-related factor 2.
Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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