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Original Paper

Luteolin Promotes Cell Apoptosis by Inducing Autophagy in Hepatocellular Carcinoma

Zhijia Cao[a] Huainian Zhang[a] Xiaoyan Cai[a,b] Wei Fang[a] Dong Chai[a] Ying Wen[a] Hongsheng Chen[a] Fujiang Chu[c] Yongli Zhang[a]

[a]Department of Biology, School of Basic Courses, Guangdong Pharmaceutical University, Guangzhou, [b]Hubei University of Medicine, Shiyan, [c]Guangdong Provincial Key Laboratory of Pharmaceutical Bioactive Substances, Guangdong Pharmaceutical University, Guangzhou, People's Republic of China

Key Words
Luteolin • Apoptosis • Autophagy • SMMC-7721 cells

Abstract
Background/Aims: Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and is a leading cause of cancer-related death worldwide. Luteolin, a flavonoid from traditional Chinese medicine, shows anti-cancer activity in many cancer cells, including HCC. However, the mechanism underlying the action of luteolin in HCC, especially its role in regulating cell autophagy, remains unclear. In the present study, we investigated the role of luteolin in regulating cell autophagy and the role of autophagy in luteolin-induced apoptosis. ***Methods:*** The 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) was used to investigate cell viability. Flow cytometry analysis was used to detect the cell cycle and cell apoptosis. Hoechst 33342 staining was used to detect cell apoptosis. Transmission electron microscopy was used to investigate autophagy. qRT-PCR and western blotting were used to detect apoptosis- and autophagy-related mRNAs and proteins. ***Results:*** Luteolin reduced the viability of SMMC-7721 cells in a time and dose-dependent manner, and induced significant G0/G1-phase arrest. In addition, the results of flow cytometry analysis and Hoechst 33342 staining showed that luteolin treatment increased the number of apoptotic cells obviously, and the results of qRT-PCR and western blotting showed that luteolin treatment increased caspase 8 and decreased bcl-2 at the mRNA and protein levels. Furthermore, luteolin increased the number of intracellular autophagosomes, promoted LC3B-I conversion to LC3B-II, and increased Beclin 1 expression. Finally, co-treatment with the autophagy inhibitor chloroquine weakened the effects of luteolin on cell apoptosis. ***Conclusion:*** Luteolin induced apoptosis in human liver cancer SMMC-7721 cells, partially via autophagy. Thus, luteolin could be used as a regulator of autophagy in HCC treatment.

Z. Cao and H. Zhang contributed equally to this work.

Yongli Zhang

Department of Biology, School of Basic Courses, Guangdong Pharmaceutical University, 280 Wai Huan Dong Road, University City of Guangzhou, (China)
Tel. +86 20 39352191; Fax +86 20 39352186; E-Mail zyl28_gdpu@163.com

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and is a leading cause of cancer-related death worldwide. In China in 2011, HCC was the fourth most common cancer and the second most common cause of death [1]. Surgery, liver transplantation, radiofrequency ablation, and chemotherapy can be used to treat HCC [2]. However, only a few early stage liver cancers can be diagnosed and treated successfully. For advanced stage HCC, there are still no suitable drugs or therapies [2]. Most treatments used for HCC are non-selective, cytotoxic molecules that have significant side effects. Therefore, it is important to find safe and effective new agents to treat HCC.

Luteolin (3',4',5, 7-tetrahydroxyflavone) is an abundant flavonoid that is present in many types of plants, including vegetables, fruits, and medicinal herbs. Plants rich in luteolin have been used in Chinese traditional medicine to treat various diseases, such as hypertension, inflammatory disorders, and cancer [3, 4]. The anti-cancer activities of luteolin have been demonstrated in many cancer cells, such as pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, and ovarian cancer [5-8]. In addition, luteolin could upregulate the mRNA expression level of intercellular adhesion molecule-1, a tumor suppressor gene [9], in mouse H22 hepatoma cells [10]. Luteolin synergizes the antitumor effects of 5-fluorouracil against human HCC cells [11]. In addition, luteolin plays a growth inhibition role in the HCC cell line HepG2 [12]. These results in HCC cells indicated that luteolin had anti-cancer activity. However, luteolin's regulatory mechanism in HCC, especially its role in regulating cell autophagy, remains unclear.

Autophagy is a catabolic process by which cytoplasmic components, such as damaged organelles, aged proteins, and damaged proteins, are sequestered by a double-membrane autophagosome, and delivered to the lysosome for degradation [13]. Basal levels of autophagy are required for cellular homeostasis. Autophagy is upregulated under various cellular stresses, such as infection, starvation, organelle damage, and protein aggregation [13, 14]. Autophagy has complicated functions in different stages of cancer [15]. On the one hand, autophagy prevents tumor initiation, acting as a suppressor pathway. On the other hand, autophagy contributes to tumor growth and progression by attenuating cellular metabolic stress, acting as a survival pathway [15]. Autophagy is regarded as a potential target for anticancer therapy [15, 16]. One of our aims was to determine whether luteolin acts as a regulator of autophagy in HCC.

Apoptosis, first described by Kerr et al., is thought to be the principal anti-cancer mechanism, and has been studied widely as a major mechanism of mediated cell death [17, 18]. However, the interplay between apoptosis and autophagy is complex and remains unclear. In this study, we investigated the effects of luteolin on cell proliferation, apoptosis, and autophagy in human hepatocellular carcinoma SMMC-7721 cells. We also studied the relationship between apoptosis and autophagy. Our results indicated that luteolin promotes cell apoptosis by inducing autophagy; thus, luteolin might be useful as a regulator of autophagy in HCC.

Materials and Methods

Cell culture and cell treatment

The human HCC cell line SMMC-7721 was purchased from the Institute of Cell Bank/Institutes for Biological Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Luteolin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) to stock concentrations of 100 mM at −20 °C.

Cell viability assay

SMMC-7721 cells were seeded into 96-well culture plates at a density of 1.5 × 10^4 cells/well in 100 μL of medium and treated with different concentrations of luteolin (0, 12.5, 25, 50, 100, and 200 μM) for 24, 48, and 72 h. Cell viability assays were carried out using a 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Cell Proliferation and Cytotoxicity Assay Kit, according to the manufacturer's instructions (Beyotime, Shanghai, China). At each time point, the MTT labeling reagent (5 mg/mL, 15 μL/well) was added, and the cells were cultured for an additional 4 h at 37 °C. The medium was aspirated, and formazan dissolved in DMSO was added (200 μL/well). The optical density (OD) of each well was measured at 490 nm using a microplate reader (multiscan MK3; Thermo Fisher Scientific, Waltham, MA, USA). Cell viability was determined using the following equation:

$$\text{Cell viability} = \frac{\text{OD of sample} - \text{OD of blank}}{\text{OD of control} - \text{OD of blank}} \times 100$$

Flow cytometry analysis for the cell cycle and cell apoptosis

The cell cycle and cell apoptosis were analyzed using a Cell Cycle Analysis Kit and an Annexin V-FITC Apoptosis Detection Kit (Beyotime), respectively, according to the manufacturer's instructions. After treatment with 25, 50, and 100 μM luteolin for 48 h, SMMC-7721 cells were harvested and washed twice with phosphate buffered saline (PBS). For the cell cycle analysis, each group of cells was fixed with 70% ethanol overnight at 4 °C. The cells were washed with PBS after centrifugation and resuspended in PBS. Then, 100 μL of RNase was added and the cells were incubated for 10 min. Propidium iodide (PI) (400 μL; 10 μg/mL) was then added, and the samples were kept in the dark for 30 min. Immediately, the cells were analyzed using a flow cytometer. For the apoptosis assay analysis, the cells were suspended in 100 μL binding buffer and stained with 5 μL annexin V-FITC and 5 μL PI for 15 min in the dark at room temperature (19-21 °C). Binding buffer (400 μL) was then added. Finally, the cells were detected and quantified using a flow cytometer within 1 h. The data were analyzed using winMDI 2.9.

Hoechst 33342 staining

Cells were seeded in 24-well culture plates overnight and then treated with 25, 50, and 100 μM luteolin for 48 h. After treatment, the attached cells were washed twice with PBS, stained with Hoechst 33342 dye (3 mM in PBS) for 10 min at room temperature, washed again, and observed under a fluorescence microscope.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Cells were treated with 25, 50, and 100 μM luteolin for 48 h, and then the total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the recommended protocol. Total RNA was reverse-transcribed to synthesize single strand cDNA using an oligo(dT) primer. Quantitative PCR was performed using the SYBR Premix Ex Taq (Takara, Dalian, China). The actin gene was used as an internal control. The primer sequences used in qRT-PCR are shown in Table 1. Gene expression was measured in triplicate and quantified using the $2^{-\Delta\Delta CT}$ method [19] normalized to the actin control.

Protein extraction and western blotting analysis

Cells were treated with 25, 50, and 100 μM luteolin for 48 h, washed twice with PBS, and re-suspended in radioimmunoprecipitation assay (RIPA) lysis buffer (RIPA: phenylmethylsulfonyl fluoride (PMSF) =100:1) for 30 min, followed by centrifugation for 10 min at 12, 000 rpm. The proteins (40 μg/well) were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat milk, the membranes were incubated with rabbit anti-LC3B (Bioss, China), rabbit anti-caspase8, rabbit anti-bcl-2, rabbit anti-beclin1, and mouse anti-β-actin (Boster) primary antibodies. After incubating for 1 h with the primary

Table 1. The primers used in this study. All primers were synthesized by Sangon (Shanghai, China)

Gene	Forward 5′-3′	Reverse 5′-3′
Actin	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG
Bcl-2	GTTTGATTTCTCCTGGCTGTCTC	GAACCTTTTGCATATTTGTTTGG
Caspase-8	GGAGGAGTTGTGTGGGGTAA	CCTGCATCCAAGTGTGTTCC
LC3B	AACCACACCCAAAGTCCTCA	CTGGTACACTGCTGCTTTCA
Beclin1	GACACTCAGCTCAACGTCAC	CTGCCACTATCTTGCGGTTC

antibodies, the membranes were washed with Tris buffered saline containing 0.05% Tween-20 (TBST) and incubated with a secondary antibody. After washing with TBST, the immunoreactive bands were visualized using the ECL Plus reagent from Pierce Thermo Scientific.

Transmission electron microscopy

Cells were treated with 25, 50, and 100 μM luteolin for 48 h. After treatment, the cells were harvested and fixed with 2.5% glutaraldehyde overnight at 4 °C. After washing with 0.1% sodium cacodylate buffer, the cells were post-fixed with 1% osmium tetroxide for 30 min, stained with 2 % uranyl acetate at 4 °C, dehydrated in a graded ethanol series, and embedded in spur resin. Ultra-thin (70 nm) sections were cut on a Reichert Ultra cut microtome, post-stained with uranyl acetate and lead citrate, and washed with water. The sections were examined by a transmission electron microscope operated at 60 kV.

Statistical analysis

The data were expressed as the mean ± SD. Statistical analysis was performed using a paired Student t-test. In all of the analyses, $P < 0.05$ was considered statistically significant.

Results

Effect of luteolin on cell viability in SMMC-7721 cells

To investigate the effect of luteolin on cell viability, SMMC-7721 cells were treated with different concentrations of luteolin and cell viability was detected by MTT assays. As shown in Fig. 1A and 1B, luteolin could reduce SMMC-7721 viability at concentrations ranging from 12.5 to 200 μM at 24, 48, and 72 h. The IC_{50} values of luteolin on SMMC-7721 cells at 24, 48, and 72 h were 103.1 μM, 46.89 μM, and 34.24 μM, respectively. In addition, the

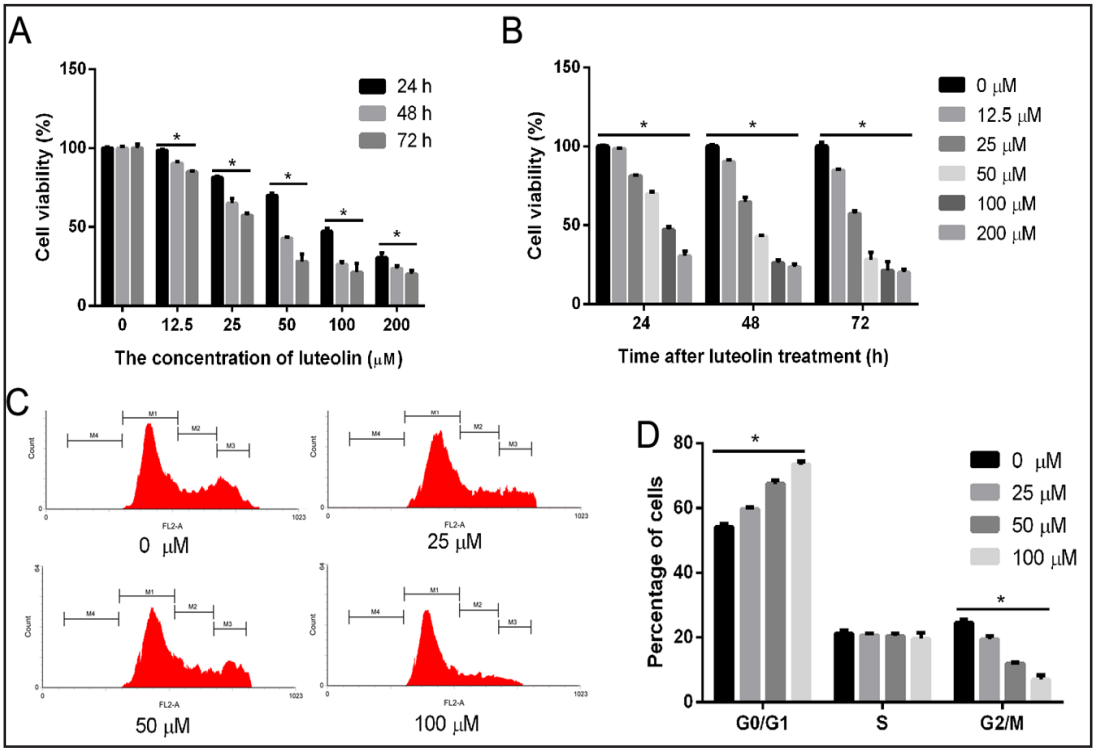

Fig. 1. Effect of luteolin on cell viability and the cell cycle. A–B: SMMC-7721 cells were treated with different concentrations of luteolin (0, 12.5, 25, 50, 100, and 200 μM) for 24, 48, and 72 h and cell viability was detected by MTT assays. C: Representative graphs of the flow cytometry analysis of the cell cycle after 25, 50, and 100 μM luteolin for 48 h. M1: G0/G1 stage， M2:S stage, M3: G2/M stage, M4: Apoptotic cell peak. D: The statistical results of flow cytometry analysis of the cell cycle. *P<0.05.

statistical analysis showed that the effect of luteolin on cell viability of SMMC-7721 cells was time-dependent and dose-dependent (Fig. 1A and 1B). To determine the mechanism by which luteolin reduces cell viability, we used flow cytometry to determine the distribution of SMMC-7721 cells in the cell cycle after treatment with 25, 50, and 100 μM luteolin for 48 h. As shown in Fig. 1C and 1D, luteolin treatment induced a significant G0/G1-phase arrest, and the percentage of cells in the G2/M-phase decreased significantly.

Effect of luteolin on cell apoptosis in SMMC-7721 cells

To investigate the effect of luteolin on cell apoptosis, SMMC-7721 cells were treated with 25, 50, or 100 μM luteolin for 48 h. Each group was then harvested for flow cytometry analysis, Hoechst 33342 staining, qRT-PCR, and western blotting. The results showed that luteolin treatment increased the percentage of early apoptosis, late apoptosis, and total

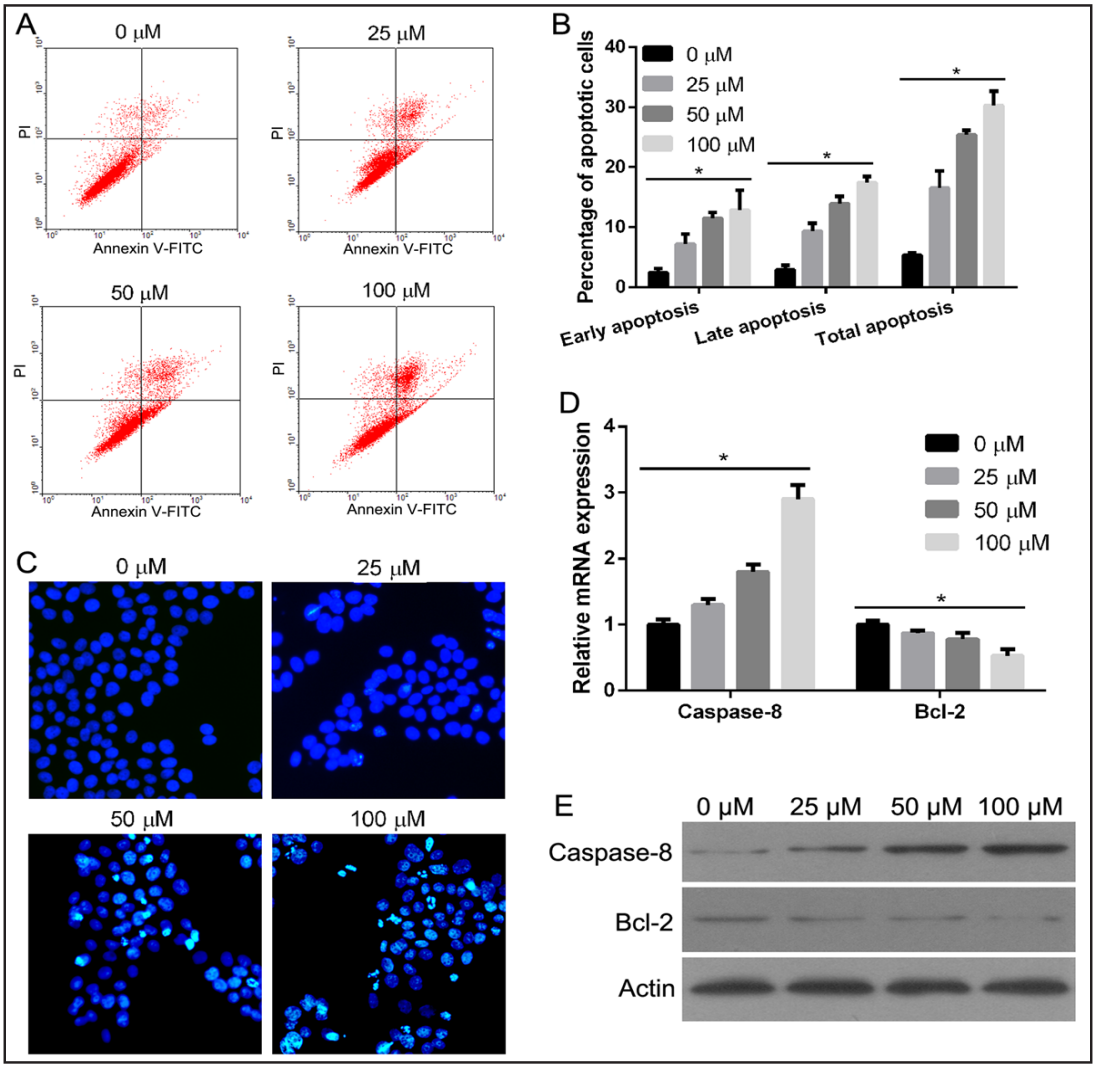

Fig. 2. Effect of luteolin on cell apoptosis. A: Representative graphs of the flow cytometry analysis of cell apoptosis after 0, 25, 50, and 100 μM luteolin for 48 h. In all four plots, viable cells are seen in the left lower quadrant ($FITC^-/PI^-$); early apoptotic cells in the right lower quadrant ($FITC^+/PI^-$); late apoptotic cells in the right upper quadrant ($FITC^+/PI^+$); and necrotic cells in the left upper quadrant ($FITC^-/PI^+$). B: The statistical results of the flow cytometry analysis of cell apoptosis. C: Representative graphs of Hoechst 33342 staining. D: The mRNA expression levels of caspase-8 and bcl-2 after luteolin treatment. E: The protein expression levels of caspase-8 and bcl-2 after luteolin treatment. *P<0.05.

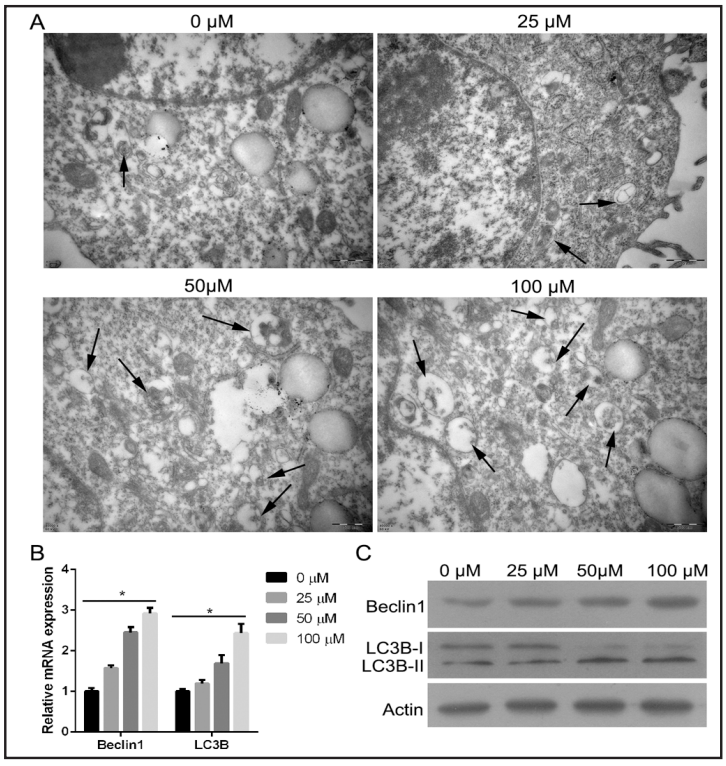

Fig. 3. The effect of luteolin on autophagy of SMMC-7721 cells. SMMC-7721 cells were treated with 0, 25, 50, or 100 μM luteolin for 48 h and harvested for transmission electron microscopy (A), qRT-PCR (B), and western blotting (C) to detect LC3B and beclin1 expression.

apoptosis in SMMC-7721 cells (Fig. 2A and 2B). In addition, the statistical analysis showed that the effect of luteolin on the apoptosis of SMMC-7721 cells was dose-dependent (Fig. 2B). Hoechst 33342 staining also indicated that luteolin treatment could increase the number of apoptotic cells (Fig. 2C). Furthermore, qRT-PCR and western blotting showed that luteolin treatment increased caspase 8 and decreased bcl-2 at the mRNA and protein levels (Fig. 2D and 2E). The statistical analysis showed that the effect of luteolin on caspase 8 and bcl-2 mRNA levels in SMMC-7721 cells was dose-dependent (Fig. 2D).

Effect of luteolin on cell autophagy in SMMC-7721 cells

To detect the effect of luteolin on SMMC-7721 cell autophagy, the formation of autophagosomes was observed using transmission electron microscopy. As shown in Fig. 3A, the number of intracellular autophagosomes after treatment with 25, 50, or 100 μM luteolin for 48 h increased compared with cells treated with 0 μM luteolin. In addition, the number of intracellular autophagosomes increased with increasing luteolin concentration. Furthermore, to determine the effect of luteolin treatment on cell autophagy, the mRNA and protein expressions of the autophagy-related genes *LC3B* and *BECN1* (beclin1) were examined using qRT-PCR and western blotting, respectively. As shown in Fig. 3B, *LC3B* and *BECN1* mRNA levels increased after treatment with 25, 50, or 100 μM luteolin and this increase was dose-dependent. The western blotting results showed that the conversion of LC3B-I to LC3B-II and beclin1 levels were increased by treatment with luteolin compared with cells treated with 0 μM luteolin.

The effect of co-treatment with luteolin and the autophagy inhibitor chloroquine on cell apoptosis

To investigate the role of autophagy in luteolin-induced cell apoptosis, the autophagy inhibitor chloroquine was used to block autophagy before luteolin treatment. The results showed that co-treatment with luteolin and chloroquine decreased the percentage of early apoptosis, late apoptosis, and total apoptosis in SMMC-7721 cells (Fig. 4A and 4B) compared with cells treated with 100 μM luteolin only. Hoechst 33342 staining also indicated that luteolin and chloroquine co-treatment decreased the number of apoptotic cells (Fig. 4C).

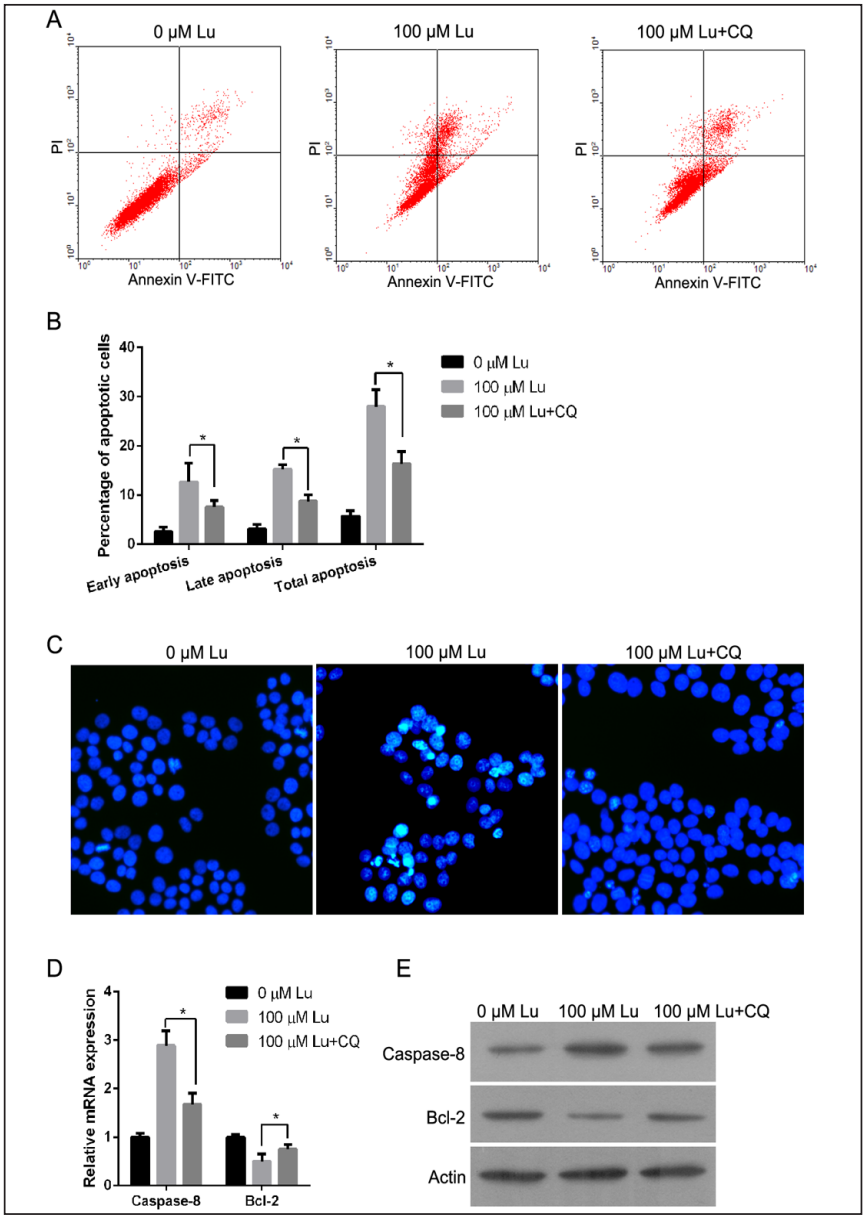

Fig. 4. Effect of luteolin (Lu) and chloroquine (CQ) co-treatment on cell apoptosis. SMMC-7721 cells were treated with 0 μM luteolin, 100 μM luteolin, and 100 μM luteolin plus CQ treatment for 48 h, and the cells were harvested for flow cytometry, Hoechst 33342 staining, qRT-PCR, and western blotting. A: Representative graphs of flow cytometry analysis of cell apoptosis. In all four plots, viable cells are seen in the left lower quadrant ($FITC^-/PI^-$); early apoptotic cells in the right lower quadrant ($FITC^+/PI^-$); late apoptotic cells in the right upper quadrant ($FITC^+/PI^+$); and necrotic cells in the left upper quadrant ($FITC^-/PI^+$). B: The statistical results of flow cytometry analysis of cell apoptosis. C: Representative graphs of Hoechst 33342 staining. D: The mRNA expression levels of caspase-8 and bcl-2. E: The protein levels of caspase-8 and bcl-2. *P<0.05.

Furthermore, qRT-PCR and western blotting showed that luteolin and chloroquine co-treatment decreased caspase 8 and increased bcl-2 mRNA and protein levels (Fig. 4D and 4E). However, the apoptosis level after luteolin and chloroquine co-treatment was still higher than that in the 0 μM luteolin-treated group.

Discussion

The antitumor activity of luteolin has been investigated in various cancer cells [6, 12, 20, 21]. For example, luteolin has been shown to induce cell cycle arrest and apoptosis, inhibit proliferation and metastatic progression, and attenuate doxorubicin-induced cytotoxicity [3, 7, 20, 22]. Although luteolin could inhibit proliferation and induce cell apoptosis in the HCC

cell line HepG2 [11, 12], its role in regulating autophagy, and the relationship between cell apoptosis and autophagy, has not been elucidated in HCC. Therefore, we focused on these issues in present study.

In present study, we found that the inhibition by luteolin of cell viability and the induction of cell apoptosis was time-dependent and dose-dependent in the HCC cell line SMMC-7721. These results were consistent with previous studies in HepG2 cells. Although these results are a repeat of the results obtained in HepG2 cells, our results further indicated that luteolin has antitumor activity in HCC. In addition, we found that luteolin treatment induced a significant G0/G1-phase arrest, which might be the mechanism by which luteolin reduces cell viability.

There are two main apoptotic pathways: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway [13, 23, 24]. The extrinsic pathway is initiated by DISC formation, Fas-associated death domain (FADD) or TRAIL-associated death domain binding to the death receptor, and pro-caspase-8 activation. The intrinsic pathway is associated with mitochondrial-mediated signals via a decrease in the mitochondrial membrane potential and the release of pro-apoptotic proteins [13, 18, 23, 24]. Bcl-2 family proteins, which include both anti-apoptotic proteins (such as bcl-2, bcl-xl, and bcl-w) and pro-apoptotic proteins (such as bax, bak, and bad), regulate the mitochondrial membrane potential and apoptosis sensitivity [13, 25]. In the present study, we found that luteolin activated caspase-8, which regulates the extrinsic pathway. In addition, luteolin also reduced the expression of anti-apoptotic protein bcl-2, which regulates the intrinsic mitochondrial pathway. Thus, we hypothesized that luteolin could regulate both the extrinsic and intrinsic pathways.

Autophagy is a lysosomal degradation pathway whereby damaged proteins and organelles are degraded to maintain cellular homeostasis [13, 26, 27]. Autophagy not only functions as a cell survival mechanism, but also can induce autophagic cell death when cells experience stress [15, 16, 28, 29]. When autophagy is induced, beclin1 and LC3 are distributed to the autophagosome membrane to regulate autophagosome formation [13, 23]. In the present study, we found that luteolin treatment not only increased the number of intracellular autophagosomes, but also increased LC3B-I conversion to LC3B-II and Beclin 1 expression. These results demonstrated that luteolin could induce autophagy in the HCC cell line SMMC-7721. This conclusion is supported by observations in other physiological process. In human osteosarcoma U2OS cells, luteolin enhanced doxorubicin-induced autophagy [30]. In addition, luteolin treatment could stimulate autophagy in a Parkinson's disease model, in post-infarction cardiac dysfunction, and in foam cell formation [31-33].

Taken together, the results of this study confirmed that luteolin treatment could stimulate both apoptosis and autophagy in HCC cells. Therefore, we predicted that luteolin might promote cell apoptosis by inducing autophagy in HCC cells. Autophagy is a double-edged sword in cancer. In contrast to the tumor-suppressor roles of autophagy, stress-activated autophagy might promote tumor cell survival [13]. Autophagy and apoptosis both function as anticancer pathways; however, the crosstalk between autophagy and apoptosis in cancer is complex [13]. In present study, we found that co-treatment with the autophagy inhibitor chloroquine weakened the effect of luteolin on cellular apoptosis. These results indicated that autophagy could activate apoptosis in luteolin treated HCC cells. On the one hand, our result conflicted with certain previous studies that indicated that autophagy could suppress apoptosis in certain physiological process of cancer [34, 35]. On the other hand, our results were supported by other studies that indicated that autophagy could promote apoptosis in certain physiological process of cancer [36, 37].

In conclusion, our results indicated that luteolin could suppress cell viability, induce G0/G1-phase arrest, and increase cellular apoptosis. These results indicated that luteolin has an antitumor effect in the HCC cell line SMMC-7721. Furthermore, luteolin could activate cellular apoptosis and co-treatment with the autophagy inhibitor chloroquine reduced the effect of luteolin on cell apoptosis, indicating that luteolin promotes cell apoptosis by inducing autophagy in HCC cells. Our study was the first to investigate the effect of luteolin

on cell autophagy and the relationship between apoptosis and autophagy in luteolin-treated HCC cells. Our results will promote further research into the use of luteolin in HCC treatment.

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Disclosure Statement

The authors declare no Disclosure Statement.

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