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ORIGINAL ARTICLE

# The critical role of quercetin in autophagy and apoptosis in HeLa cells

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**Abstract** In recent years, the effects of quercetin on autophagy and apoptosis of cancer cells have been widely reported, while effects on HeLa cells are still unclear. Here, HeLa cells were subjected to quercetin treatment, and then proliferation, apoptosis, and autophagy were evaluated using MTT, flow cytometry, and MDC staining, respectively. The LC3-I/II, Beclin 1, active caspase-3, and S6K1 phosphorylation were detected using Western blot assay. The ultrastructure of HeLa was observed via transmission electron microscope (TEM). Our findings showed that quercetin can dose-dependently inhibit the growth of HeLa cells. The MDC fluorescence was enhanced with increased concentration of quercetin and hit a plateau at 50 μmol/l. Western blot assay revealed that LC3-I/II ratio, Beclin 1, and active caspase-3 protein were enforced in a dose-dependent method. However, the phosphorylation of S6K1 gradually decreased, concomitant with an increase of autophagy. In addition, TEM revealed that the number of autophagic vacuoles was peaked at 50 μmol/l of quercetin. Besides, interference of autophagy with 3-MA led to proliferation inhibition and increased apoptosis in HeLa cells, accompanied by the decreased LC3-I/II conversion and the increased active caspase-3. In conclusion, quercetin can inhibit HeLa cell proliferation and induce protective autophagy at low concentrations; thus, 3-MA plus quercetin would suppress autophagy and effectively increased apoptosis.

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## Introduction

Quercetin exists in flavonoids of a variety of plants, sharing robust antioxidant and anticancer properties [1], and has been widely investigated as the therapy of different cancers [2]. Our previous findings identified quercetin can inhibit proliferation and induce apoptosis in HeLa cells [3]. Quercetin alone or quercetin combined with cisplatin have an inhibitive effect on the adhesion, migration, and invasion of HeLa cells [4]. However, the specific mechanisms of autophagy in HeLa cells were not well characterized.

Autophagy phenomenon widely exists in eukaryotic cells and is of great importance in maintaining a stable microenvironment of cells. On the other hand, deregulatory autophagy was associated with tumorigenesis, and has been recommended as antitumor therapy. More and more studies have shown that autophagy activity was altered in a variety of human tumor cells [5–7]. It should be noted that autophagy may also have antitumor effect in some circumstances, whereas autophagy can also protect tumor cells from destruction under certain conditions. Thus, it is essential to explore the detailed role of autophagy in cervical cancer.

In this work, HeLa cells were subjected to quercetin treatment, and then proliferation, apoptosis, and autophagy was evaluated using MTT, flow cytometry, and MDC staining, respectively. The LC3 conversion, Beclin 1, active caspase-3, and S6K1 phosphorylation were detected using Western blot assay. The ultrastructure of HeLa was observed via transmission electron microscope.

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## Materials and methods

### Cell culture

HeLa cell line was donated by the Medicine School of Wuhan University. HeLa cells with 10 % fetal bovine serum, 100 U/ml penicillin, RPMI-1640 medium (American Gibco Company) with 100 mg/l streptomycin, placed into closed incubator thermostat with 5 % $CO_2$, 37 °C, 95 % humidified culture and passage. EDTA digestive trypsin digestion, passaged every 2 to 3 days. Cells used for experiments were in logarithmic growth phase. Quercetin was purchased from Chengdu Superman Sik Corp. Antiactive caspase-3 antibody, anti-p-S6K1 (T389) antibody, and anti-S6K1 antibody were obtained from British Abcam Company; antimicrotubule-associated protein 1 light chain 3 (LC3)A/B and anti-Beclin 1 antibody were bought from Cell Signaling Technology; and Annexin V-FITC/PI apoptosis detection kit was purchased from Nanjing KGI biological Technology Development Co., Ltd.

### Tetrazolium blue assay

After trypsin digestion, HeLa cells were adjusted at a concentration of 5×105 pieces/ml, and then were seeded in 96-well plates with 200 μl per well of cells. We divided cells into six groups following 24-h culture: negative control group (RPMI-1640 medium alone), solvent control group (medium plus DMSO), and the rest of four experimental groups (quercetin 12.5, 25, 50, and 100 μmol/l, respectively). Culture medium was discarded after 24-h incubation and added 20 μl of tetrazolium blue (MTT, 5 mg/ml). After 4-h incubation, add 150 μl of DMSO was further appended, and oscillate for 10 min and then subjected to the microplate reader at 492-nm wavelength. The experiment was repeated for three times. Inhibition rate of cell proliferation was calculated as (1-A test hole/A solvent control wells)×100 %.

### MDC staining

MDC staining method was conducted as reported by Biederbick [8]. HeLa cells were harvested in logarithmic growth phase into single-cell suspension with 0.25 % trypsin digestion, and adjusted into 1.0×104 cells/ml. Then, cells were subjected to quercetin with concentration of 12.5, 25, 50, and 100 μmol/l), and DMEM or/and solvent DMSO were added to the control group, respectively. After 24-h treatment, cells were incubated into fresh DMEM containing MDC (50 μmol/l) with 37 °C, 5 % $CO_2$ for 1 h. And then, cells were fixed after 30 min by 4 % paraformaldehyde and observed in inverted fluorescence microscope.

### Western blot

Total proteins were extracted from cells and quantified using the BSA method. After 10 % SDS-PAGE electrophoresis, proteins were transferred to a membrane and closed in 5 % skim milk at room temperature for 1 h, followed by adding a primary and secondary antibody. The membrane was washed. Enhanced chemiluminescent detection (ECL) was performed to detect the expression quantity of protein. The film is analyzed using AlphaEase software (Alpha Innotech American products). The experiment was repeated for three times.

### TEM

TEM detection steps are as follows:

1. Fixation: cells were fixed with 2.5 % glutaraldehyde for 2 h, rinsed by 0.1 M phosphate for three times, 15 min for each time. Then, cells were immersed in 1 % osmium tetroxide for 3 h and rinsed by 0.1 M phosphate for three times, 15 min for each time.
2. Dehydration: cells were sequentially immersed at 4 °C in the sequential 50, 70, and 90 % ethanol for 15 min; immersed in 90 % ethanol and 90 % acetone solution for 15 min; and then immersed in 90 % acetone for 15 min; finally use 100 % acetone to dehydrate three times at room temperature, 15 min for each time.
3. Embedding: cells were treated at room temperature, with 100 % acetone plus embedding solution (2:1), for 3 h; and then treated with 100 % acetone plus embedded solution (1:2) overnight; and then with 100 % embedding solution for 3 h.
4. Curing: specimens were treated at 37 °C for 24 h; 45 °C for 12 h; 60 °C for 24 h sequentially.
5. Slice: slice thickness of 50 nm was conducted.
6. Observation: we used 3 % uranyl acetate and led citrate for double staining, and then used TEM to observe autophagosome and radiography.

### Statistical analysis

All data were analyzed in SPSS l7.0 statistical analysis software, and measurement data was expressed as mean±standard deviation (mean±SD). The differences among groups were compared using univariate analysis of variance, and differences between the two groups were compared using Student-Newman-Keuls. $p<0.05$ considered statistically significant.

## Results

### Quercetin inhibits proliferation of HeLa cells

To figure out the effect of quercetin on HeLa cells, we carried out MTT assay and found that HeLa cell proliferation was inhibited in a dose-dependent manner. Inhibition rate of proliferation of HeLa cell administrated with 12.5, 25, 50, and 100 μmol/l of quercetin was 26.2±6.8, 39.8±11.4, 48.5±9.1, and 79.1±6.4 %, respectively. The differences from the control group were statistically significant ($p<0.01$).

### Quercetin affected autophagy of HeLa cells

As is known to all, MDC is phagocytosed and gathered in autophagic vesicles, and can be observed under the fluorescence microscope. Fluorescence-infected MDC autophagic vesicles showed spotty structures scattered in the nucleus of HeLa cells. In the present study, little sporadic punctate fluorescent particles can be seen in the cytoplasm of HeLa cells treated by 12.5 μmol/l of quercetin. By contrast, cells with 25 μmol/l of quercetin contained dot-like phosphor particles and fluorescent particles. Besides, cells treated by 50 μmol/l of quercetin generated much more punctate fluorescent particles than those mentioned above. Conversely, when cells were treated with 100 μmol/l of quercetin, there were less dot-like phosphor particles than those with 50 μmol/l of quercetin. As expected, MDC staining fluorescence intensity is very weak and rarely showed punctate structures in the two control groups. These results suggested that the administration with 50 μmol/l of quercetin can induce the strongest autophagy capacity in HeLa cells.

### Quercetin affected the relevant proteins in HeLa cells

To further elucidate the molecular mechanisms of quercetin on HeLa cells, we conducted Western blot to detect the relevant proteins. As shown in Table 1, there is no significant statistically differences in the two control groups ($p>0.05$). Notably, we found that the LC3-I conversion into LC3-II can occur in a concentration-dependent manner, which is consistent with results from the MDC fluorescence. Conversion rate of LC3-I conversion to LC3-II was peaked at 50 μmol/l of quercetin compared with the other concentrations ($p<0.05$). The protein expression of Beclin 1 increased with the increasing of quercetin concentrations (less than 50 μmol/l), but decreased at a concentration of 100 μmol/l. All of these demonstrated that HeLa cells had the highest levels of autophagy at low concentrations of quercetin (50 μmol/l or less). Next, we used Western blot to detect the level of phosphorylation of S6K1. The p-S6K1 (T389) levels gradually decreased, accompanied by the increased autophagy (compared with the control group, $p<0.05$). In addition, the expression of active caspase-3 also increased with the increasing of quercetin concentrations, suggesting that levels of apoptosis increased with increasing concentration.

### Cell ultrastructure is observed by transmission electron microscopy

Using transmission electron microscopy, we detect ultrastructures of HeLa cells under different concentrations of quercetin. Morphology of cells in negative control group was normal and did not altered significantly. After 24-h treatment by quercetin of 50 μmol/l, a large number of vacuoles in the cytoplasm were observed, indicating a lot of autophagy and lysosomal autophagy were generated (Fig. 1).

### The effect of quercetin on autophagy and apoptosis in HeLa cells

When autophagy was inhibited by 3-MA, we found that inhibition rates of autophagy in different treatment groups were: 0 in negative control group 7.5±1.3 % in 3-MA group, 48.5±9.1 % in quercetin group (50 μmol/l), and 71.2±9.4 % in 3-MA plus quercetin group (Fig. 2). In addition, apoptotic rates of HeLa cells were 2.8±0.5 % in negative control group, 6.5±0.7 % in 3-MA group, 46.3±3.8 % in the quercetin group (50 μmol/l), and 65.4±4.6 % in 3-MA plus quercetin group (Fig. 3). The expression proportion of LC3-I/II showed 1.15±0.14 in the quercetin group (50 μmol/l) and 0.41±0.09 in 3-MA plus quercetin group, both of which were statistically significantly different ($p<0.01$). These data suggested that 3-MA abrogated autophagy induced by quercetin. The expression of active caspase-3 was higher in 3-MA plus quercetin group (1.12±0.10) than that in quercetin alone group

**Table 1** Protein expression in HeLa cells treated by different concentrations of quercetin

| | LC3-II/I | Beclin 1 | p-S6K1/S6K1 | Active caspase-3 |
|---|---|---|---|---|
| Blank control | 0.28±0.10 | 0.32±0.03 | 0.85±0.11 | 0.11±0.03 |
| Solvent control | 0.29±0.11 | 0.35±0.04 | 0.89±0.14 | 0.15±0.03 |
| 12.5 μmol/l | 0.46±0.09 | 0.52±0.06 | 0.57±0.09 | 0.37±0.04 |
| 25 μmol/l | 0.74±0.12 | 0.73±0.05 | 0.42±0.10 | 0.56±0.05 |
| 50 μmol/l | 1.15±0.14 | 0.92±0.07 | 0.26±0.05 | 0.83±0.07 |
| 100 μmol/l | 0.53±0.11 | 0.49±0.05 | 0.39±0.07 | 1.04±0.10 |

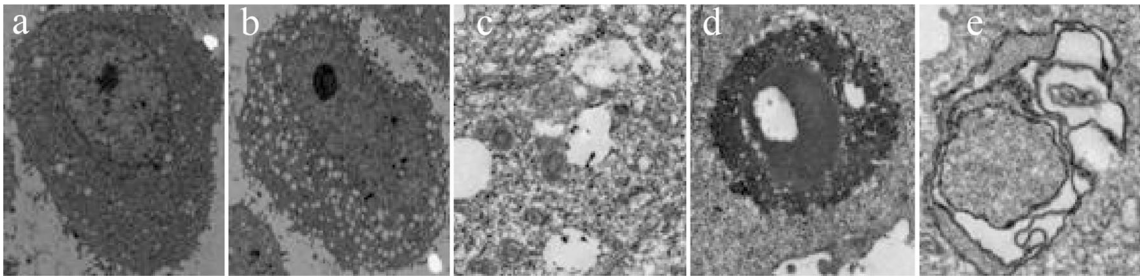


**Fig. 1** Cell ultrastructure is observed by transmission electron microscopy. **a** Negative control cells, *scale*=2 μm; **b** 50 μmol/l of quercetin after cell scale=2 μm; **c** 50 μmol/l of quercetin, cytoplasm autophagy and lysosomalautophagy can be observed, scale=0.5 μm; **d** lysosomal autophagy were shown, ruler=200 nm; **e** autophagosome was showed, ruler=200 nm

(50 μmol/l) (0.83±0.07), suggesting that apoptosis induced by quercetin increased with the aid of 3-MA.

## Discussion

In recent years, some reports demonstrated the effects of quercetin on the induction of autophagy in tumor cells. Liang et al. identified that quercetin can induce autophagy in bladder cancer BIU-87 cell and in early stage, autophagy induced by quercetin can protect BIU-87 cells, and delay apoptosis [9]. Quercetin was used to inhibit proteasome activity of breast cancer cells MCF-7, and verified intracellular proteasome was involved in quercetin-induced autophagy [10]. As for HeLa cells, there was few report to identify the role of quercetin in autophagy. Our study investigated the effect of different concentrations of quercetin on cell proliferation using MTT and found that with the increase of quercetin concentrations, the inhibition rate of proliferation of HeLa cells increased in a dose-dependent manner. We further observed lysosomal autophagy using MDC staining and explored the ultrastructure of HeLa cells by TEM. Our result showed that the level of autophagy increased with the increase of quercetin concentration, and maximized at 50 μmol/l, indicating quercetin enhanced the autophagy capacity of HeLa cells.

Subsequently, we investigated the expression profile of autophagy-related proteins when HeLa cells were subjected to quercetin treatment. In this study, LC3 acts as an autophagosome membrane marker and is involved in the formation of the autophagy body. There are two kinds of LC3 protein in cells, including LC3-I and LC3-II, among which LC3-I/II conversion reflects the number of autophagosome [11]. Beclin 1 is also specific gene involved in mammalian autophagy, combined with phospholipids inositol triphosphate-kinase (PI3K), and involved in the formation of autophagosomes. Wang et al. reported that the overexpression of Beclin 1 in HeLa cells can increase the intracellular levels of autophagy, showing that the cell growth and tumorigenic potential in nude mice were significantly inhibited [12]. In this study, the level of LC3-I/II conversion, Beclin 1 protein gradually increased with increasing concentrations of quercetin and reaching a maximum at 50 μmol/l instead of 100 μmol/l, indicating that low concentrations of quercetin can induce occurrencce of protective autophagy in HeLa cells, but autophagy level decreased in high concentrations.

It is believed that autophagy was controlled by mTORC signaling pathway, which includes mTORC1 and mTORC2.

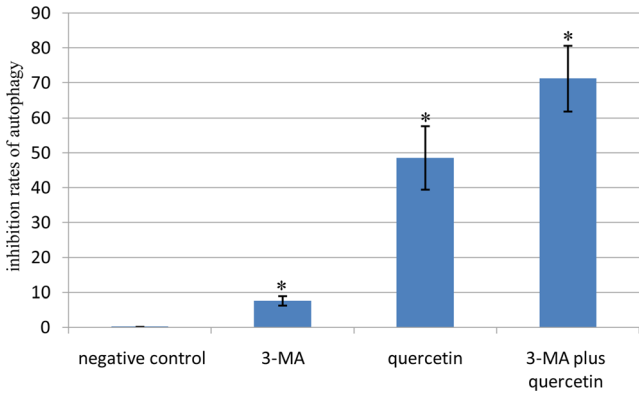


**Fig. 2** The effect of quercetin on autophagy in HeLa cells. Cells were subjected to the different treatment as mentioned in the "Materials and methods." *Each bar* represents the mean±SD of three independent experiments; *$p<0.001$, compared with control, one-way ANOVA or Student-Newman-Keuls

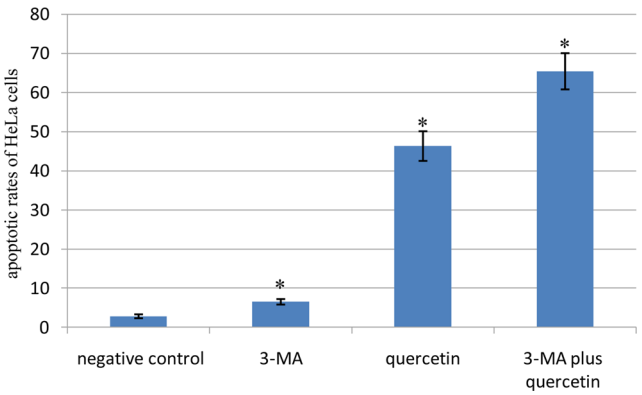


**Fig. 3** The effect of quercetin on apoptosis in HeLa cells. Cells were subjected to the different treatment as mentioned in the "Materials and methods." *Each bar* represents the mean±SD of three independent experiments; *$p<0.001$, compared with control, one-way ANOVA or Student-Newman-Keuls

Phosphorylation activation of downstream substrates is the major activation of mTORC1 signaling pathway. Ribosomal protein S6 kinase 1 (S6K1) is a key substrate for the activation of the phosphorylation levels of mTORC1 signaling pathway [13]. In order to investigate whether mTORC 1 pathway is involved in the regulation of autophagy in HeLa cells induced by quercetin, Western blot was used to detect the phosphorylated protein expression levels of S6K1 (p-S6K1) and S6KI in HeLa cells. S6K1 phosphorylation levels decreased in a dose-dependent manner (less than 50 μmol/l), indicating that quercetin induced autophagy by negative regulation of mTORC1.

The study found that protein levels of active caspase-3 gradually increased with increasing of quercetin concentrations, suggesting that quercetin induced apoptosis through mitochondrial pathway in HeLa cells. Autophagy and apoptosis are obviously different in terms of morphological or biochemical metabolic pathways, but there is a link between their functions. Due to interaction of apoptosis and autophagy-related molecules, in some cases, autophagy and apoptosis can promote or antagonize each other [14, 15]. Our results suggest that the proliferation rate of HeLa cell in 3-Ma and quercetin group is lower than that in quercetin alone group, indicating that inhibition of autophagy can increase the inhibition rate of HeLa cells. Consistent with these results, previous results from Annexin V-FITC/PI staining and flow cytometry revealed that apoptosis rate in 3-MA and quercetin group was higher than quercetin alone group, indicating that inhibition of autophagy can promote apoptosis of HeLa cells.

In conclusion, quercetin can induce apoptosis and protective autophagy in HeLa cells at low quercetin concentrations. Autophagy induced by quercetin is negatively regulated by mTORC1, and apoptosis can be greatly promoted by quercetin and autophagy inhibitors. Therefore, the application of quercetin in the treatment of cancer may open a new door for the treatment of cancer.

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**Conflicts of interest** None

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