## Original Article The chemosensitization effect of quercetin on cisplatin induces the apoptosis of human colon cancer HT-29 cell line

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**Abstract:** Objectives: The present study is to investigate the effect of quercetin on cisplatin-induced growth inhibition, apoptosis and nuclear factor-kappa B (NF-κB) expression in colon cancer HT-29 cells. Methods: HT-29 cells were treated with quercetin, cisplatin or the combination of both. MTT assay was applied to examine cell viability. Transmission electron microscopy and laser scanning confocal microscopy were used to observe morphological changes of HT-29 cells. Flow cytometry was employed to determine the apoptosis rate of different groups. Immunohistochemical analysis was introduced to measure the expression level of NF-κB. Results: MTT assay showed that the combination of both drugs had the strongest inhibitory effect on cell growth. Cells in the combination group showed typical apoptotic morphological changes under the microscope. Flow cytometry showed that the apoptosis rate in the combination group was the highest, and the cell cycle was blocked in  $G_2/M$  phase. Immunohistochemistry showed that NF-κB expression in the combination group was significantly lower than that in cisplatin group. Conclusions: Quercetin enhances cisplatin-induced apoptosis of human colon cancer HT-29 cells. The possible mechanism is partially the inhibition of the activation of NF-κB expression, suggesting that quercetin in combination with cisplatin provides a possibly effective approach in the treatment of patients with colon cancer.

Keywords: Quercetin, cisplatin, colon cancer HT-29 cell line, nuclear factor-kappa B

#### Introduction

Colon cancer is one of the most common malignant tumors in China. Surgical resection is currently the best approach to cure colorectal cancer. In addition, post-surgical adjuvant chemotherapy further improves the curative effect through reducing the risk of relapse and metastasis. Drug resistance, relapse and metastasis are the three most important causes contributing to clinical treatment failure and death. Therefore, it is urgently needed to develop neoadjuvant chemotherapeutic drugs to enhance clinical efficacy [1, 2]. Cisplatin is a commonly used anti-tumor chemotherapeutic drug, but its clinical efficacy is not always good due to multidrug resistance and side effects. Previous researches demonstrate that chemotherapy drugs activate nuclear factor-kappa B (NF-kB) in tumor cells, which reduces their clinical efficacy [3, 13], suggesting that NF-kB activation might be involved in the drug resistance of tumor cells [4]. Therefore, inhibition of NF-κB activation induced by chemotherapy drugs can possibly enhance the curative efficacy. Quercetin is a flavonoid widely distributed in a variety of plants, flowers, leaves and fruits. Quercetin can be easily extracted, separated and detected. Recent researches reveal that quercetin inhibits the proliferation of tumor cells, reverses multidrug resistance, enhances the sensitivity of other anticancer drugs [5], and plays an anti-tumor effect by inhibiting NF-κB activation. In the present study, we investigate the effect of quercetin combined with cisplatin on human colon cancer HT-29 cell line, as well as its biological mechanism.

## Materials and methods

#### Cells

Colon cancer HT-29 cells (Shanghai Xinyu Biological Technology Co., Ltd., Shanghai, China)

were conventionally cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated calf serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 100 U/ml benzyl penicillin and 100  $\mu$ g/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified environment with 5% CO<sub>2</sub> at 37°C. Cells in logarithmic growth phase were used in experiments.

## MTT assay

Cells in logarithmic growth phase were seed ed into 96-well plates at 1×10<sup>4</sup> cells/well. After 24 h, the supernatant culture medium was refreshed and the indicated concentrations of quercetin (12.5, 25, and 50 µmol/L; Sigma-Aldrich, St. Louis, MO, USA), cisplatin (0.1, 1, and 10 mg/L; Sigma-Aldrich, St. Louis, MO, USA), or the combination of the two drugs (quercetin, 50 µmol/L; cisplatin, 10 mg/L) were added, with 4 wells in each group. For control, only vehicle solvent was added. The cells were incubated for 12 h, 24 h, and 48 h, respectively. Then, 20 µL of 5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) was added before incubation at 37°C for 4 h. MTT was removed and 150 µL of dimethyl sulfoxide was used to disolve the formazan product. After gently shaking for 10 min, the absorbance was determined at 570 nm using an automated microplate reader (DNM-9606, Perlong Medical Instruments, Beijing, China).

## Transmission electron microscopy

Cells treated with drugs for 24 h were collected, washed with phosphate-buffered saline (PBS), and immediately fixed with glutaraldehyde at 4°C. Then, the cells were mixed with a pipette to make cell clusters floating, fixed with osmic acid for 2 h, dehydrated with alcohol, permeated, epoxy resin-embedded, followed by slicing and staining with uranium lead. Finally, the cells were observed under transmission electron microscope (JEM100CX, JEOL, Tokyo, Japan) for imaging.

## Laser scanning confocal microscopy

Cells treated with drugs for 24 h were collected, washed with PBS, mixed thoroughly with a pipette, and stained with acridine orange. The morphology of the cells was observed with laser scanning confocal microscope (LeicaSP2, Leica, Wetzlar, Germany) for imaging.

## Flow cytometry

Cells treated with drugs for 24 h were collected, washed with PBS, fixed with 70% ethanol, and stored at 4°C overnight. After centrifugation, the cells were precipitated to remove fixing liquid, and then the cells were incubated with RNaseA and propidium iodide for 30 min in the dark. The samples were analyzed by flow cytometry (EPICSXL, Beckman Coulter, Brea, CA, USA) within 1 h to detect the apoptosis rate and cell cycle distribution.

## Immunohistochemistry staining

Cells were grown on sterilized coverslips which were placed in 12-well plates with 1×10<sup>6</sup> cells/ well in 100 µL. The supernatant culture medium was refreshed after 24 h culture. Cisplatin (10 mg/L) and the combination of cisplatin with quercetin were administrated, respectively. Cells in control group were treated only with vehicle solvent. After 10 h treatment, the cells were washed with PBS, fixed with acetone at 4°C, dried naturally and stored in wet box at 4°C. Streptavidin-perosidase (SP) immunohistochemistry staining and diaminobenzidine (DAB) color reaction were performed (Fuzhou Maixin Biological Technology Development Company, Fuzhou, China). The cells were counterstained with hematoxylin, dehydrated, pellucidum, mounted with neutral gum, and observed under the microscope before imaging. A total of 2,000 cells were counted randomly under high power field in each picture, and the percentage of cells with positively stained nucleus was calculated. Cells with positive staining by anti-rabbit NF-kBp65 polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) showed brown particles in nucleus or cytoplasm, but mainly in nucleus.

## Statistical analysis

All results were expressed as means  $\pm$  SD from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical significance was assessed by one way analysis of variance (ANOVA) using SPSS 13.0 (IBM, Armonk, NY, USA). A *P*-value of less than 0.05 was considered statistically significant.

Groups	Concentration	Absorbance		
		12 h	24 h	48 h
Control	0	0.278 ± 0.016	0.489 ± 0.020	0.799 ± 0.043
Quercetin (µmol/L)	12.5	0.279 ± 0.014	0.496 ± 0.013	0.790 ± 0.016
	25	0.279 ± 0.014	0.492 ± 0.028	0.779 ± 0.023
Cisplatin (mg/L)	50	0.272 ± 0.011	0.430 ± 0.058	0.738 ± 0.038*
	0.1	0.278 ± 0.012	0.498 ± 0.020	0.788 ± 0.012
	1	0.278 ± 0.015	0.488 ± 0.136	0.770 ± 0.011
	10	0.259 ± 0.015	0.393 ± 0.087	0.639 ± 0.038**
Quercetin (µmol/L) + cisplatin (mg/L)	50 + 10	0.259 ± 0.014	0.298 ± 0.096**	0.311 ± 0.018**

 Table 1. Inhibitory effect of quercetin, cisplatin or the combination of both on colon cancer HT-29 cell proliferation

Note: \**P* < 0.05; \*\**P* < 0.01 compared with control.



**Figure 1.** Transmission electron microscopy of HT-29 cells in (A) control group, (B) cisplatin group, (C) quercetin group, and (D) combination group. The cells were cultured for 24 h before experiments. Magnification, ×6000.

#### Results

# The combination of quercetin and cisplatin strongly inhibits HT-29 cell growth in vitro

To test the effect of quercetin and cisplatin on *in vitro* HT-29 cell growth, we measured the optical density of cells at 12, 24 and 48 h after treatment with quercetin, cisplatin or the combination of both using MTT assay. The data showed that cell growth was significantly inhibited by quercetin (50  $\mu$ mol/L) at 48 h, or cisplatin (10 mg/L) at 48 h compared with control (P < 0.05). Of note, the growth of cells treated

with both quercetin (50  $\mu$ mol/L) and cisplatin (10 mg/L) was more significantly inhibited compared with control at both 24 h and 48 h (P < 0.01) (**Table 1**). The result suggests that the combination of quercetin and cisplatin strongly inhibits HT-29 cell growth *in vitro*.

Treatment with the combination of quercetin and cisplatin alters the morphology of HT-29 cells

To study the effect of the drugs on cell morphological changes, transmission electron microscopy and laser scanning confocal microscopy

## Quercetin and cisplatin on colon cancer cells





**Figure 2.** Laser scanning confocal microscopy of (A) DNA and (B) RNA in control group, (C) DNA and (D) RNA in cisplatin group, (E) DNA and (F) RNA in quercetin group, and (G) DNA and (H) RNA in the combination group. HT-29 cells were cultured for 24 h before acridine orange staining. Green fluorescence indicates DNA, while red fluorescence indicates RNA. Magnification, ×400.

were performed. Transmission electron microscopy showed that the cell membrane in control group was complete, and cell structures such as nucleus and organelles were clearly visible (Figure 1). After treatment with the combination of drugs, karyopyknosis appeared, chromatin was condensed to crescent or ring shapes under nuclear membrane, cytoplasm was condensed, endoplasmic reticulum became loose and fused with cell membrane, and vacuole was formed, and at advanced stage, the nucleus was fragmented and apoptotic bodies appeared (Figure 1). Laser scanning confocal microscopy showed that the nucleus morphology of cells in control group was plump, stained DNA showed evenly dispersed green fluorescence, and stained RNA showed red fluorescence (Figure 2). After treatment with the combination of drugs, the nucleus volume was decreased, chromatin was condensed to granular or ring shapes under nuclear membrane, and the green fluorescence was significantly enhanced, and at advanced stage, cell membrane blebbing and apoptotic body formation were observed, and fluorescence was decreased (Figure 2). These results indicate that treatment with the combination of quercetin and cisplatin alters the morphology of HT-29 cells.

Treatment with the combination of quercetin and cisplatin dramatically enhances apoptosis and the ratio of HT-29 cells in  $G_{a}/M$  phase

To determine the effect of quercetin and cisplatin on cell cycle distribution and apoptosis, flow cytometry was employed. After treatment for 24 h, the percentage of apoptotic cells in the combination group was significantly higher than that in control (P < 0.01), and that in cisplatin group was also significantly higher than that in control (P < 0.05). In addition, the ratio of cells in G<sub>2</sub>/M phase was significantly increased in the combination group compared with control (P < 0.01), indicating that the cell cycle was arrested in the G<sub>2</sub>/M phase (Figure 3; Table 2). These results suggest that treatment with the combination of quercetin and cisplatin dramatically enhances apoptosis and the ratio of HT-29 cells in  $G_2/M$  phase.

Cisplatin activates HT-29 cells by enhancing the expression of NF- $\kappa$ B, while treatment with the combination of quercetin and cisplatin inhibits the expression of NF- $\kappa$ B activated by cisplatin

To examine the effect of cisplatin and the combination of cistplatin and quercetin on the



**Figure 3.** Effect of quercetin, cisplatin or the combination of both on the apoptosis and cell cycle of colon cancer HT-29 cells in (A) control group, (B) cisplatin group, (C) quercetin group, and (D) combination group. The cells were cultured for 24 h before experiments. Flow cytometry was performed to examine apoptosis and cell cycles.

**Table 2.** Effect of quercetin, cisplatin or the combination of both on the apoptosis and cell cycle of colon cancer HT-29 cells (means  $\pm$  SD. %, n = 4)

Groups Apoptosis $G_0/G_1$ S $G_2/M$	
Control $1.7 \pm 0.6$ $57.6 \pm 2.8$ $33.0 \pm 2.8$ $8.4 \pm 1.$	2
Quercetin $4.5 \pm 2.2$ $58.9 \pm 4.6$ $31.6 \pm 5.0$ $9.9 \pm 2.$	0
Cisplatin $8.5 \pm 2.0^*$ $52.8 \pm 4.6$ $37.1 \pm 3.0$ $10.2 \pm 1$	.9
Quercetin + Cisplatin $14.3 \pm 2.6^{**}$ 50.2 ± 6.0 $34.8 \pm 5.2$ $13.8 \pm 0.8$	3**

Note: \*P < 0.05; \*\*P < 0.01 compared with control.

expression of NF-κB protein in HT-29 cells, SP immunohistochemical staining was performed. The data showed that the positive rate of NF-κB nuclear staining in cisplatin group was  $63.8 \pm 5.6\%$ , which was significantly higher than that in combination group  $23.2 \pm 2.3\%$  (P

< 0.01) (Figure 4). This result indicates that cisplatin activates HT-29 cells by enhancing the expression of NF- $\kappa$ B, while treatment with the combination of querce-tin and cisplatin inhibits the expression of NF- $\kappa$ B activated by cisplatin.

### Discussion

Induction of tumor cell apoptosis is one of the main mechanisms of many chemotherapeutic drugs to exert anti-tumor effect [6]. The transcriptional activation of NF- $\kappa$ B expression by chemotherapeutic drugs is the main mechanism of tumor to induce resistance to chemo-





Figure 4. Effect of quercetin, cisplatin and the combination of both on NF-kB protein expression in human colon cancer HT-29 cells. A. HT-29 cells in control group; B. HT-29 cells treated with cisplatin (10 mg/L) for 10 h; C. HT-29 cells treated with quercetin (50 µmol/L) for 10 h; D. HT-29 cells treated with the combination of quercetin (50 µmol/L) and cisplatin (10 mg/L) for 10 h. SP immunohistochemical staining was used to measure NF-kB protein expression. Magnification, ×400. E. Quantification of the percentage of NF-κB-positive cells. \*P < 0.05 compared with control; \*\*P < 0.01 compared with combination group.

therapy. Inhibition of the activation of NF- $\kappa$ B expression may enhance the efficacy of chemotherapy [7]. Quercetin is widely distributed in a variety of vegetables, fruits and herbs, and it almost has no toxicity to the human body. Quercetin can induce apoptosis of cancer cells when used alone [8-10]. Recent researches have discovered that quercetin induces tumor cell apoptosis through inhibiting the activation of NF- $\kappa$ B expression [11]. To further study whether it enhances the efficacy of chemotherapy through inhibiting the activation of NF- $\kappa$ B expression, and to seek new approaches to overcome drug resistance of chemotherapeutic

drugs, we chose human colon cancer HT-29 cell line as the experimental model. In the present study, the expression of NF-kB was detected using immunocytochemistry method. The experiment result reveals that the expression of NF-kB is increased when treated with 10 mg/L cisplatin for 10 h alone, but is decreased significantly when treated with both quercetin  $(50 \mu mol/L)$  and cisplatin (10 mg/L) for 10 h. Consistent with these results, more evidence has been published to confirm that quercetin enhances the therapeutic effect of cisplatin by inhibiting the activation of NF-KB expression. For example, Cipak et al. [12] report that HL60 and LI210 cell lines pretreated with quercetin show a higher apoptosis rate induced by cisplatin, and the combination of quercetin and cisplatin has a synergistic effect. In summary, due to its low toxicity, quercetin may be used in clinical care, locally or systematically, as a promising and safe therapeutic approach.

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## Disclosure of conflict of interest

None.

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