STUDY ON THE EFFECT OF URSOLIC ACID COMBINED WITH OXALIPLATIN ON THE GROWTH OF RECTAL CANCER AND ITS POSSIBLE MECHANISM

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ABSTRACT

Objective: *To investigate the effect of ursolic acid combined with oxaliplatin in inhibiting cell growth in rectal cancer and the possible mechanism.*

Methods: *Human rectal cancer cell line SW480 was cultured in vitro, and all cells were divided into control group (with culture solution only), ursolic acid group (45 μmol/L ursolic acid treatment), oxaliplatin group (10mg/L oxaliplatin treatment) and combination group (45 μmol/L ursolic acid +10mg/L oxaliplatin treatment). The changes of cytological morphology after drug treatment were observed under inverted microscope. The change of cell proliferation level was detected by MTT colorimetry. The changes of cell cycle and apoptosis rate were detected by flow cytometry. The protein expressions of Bax and Bcl-2 were detected by Western blot.*

Results: *The number of cells in ursolic acid group and oxaliplatin group decreased, the morphology became round, and the growth was inhibited. The cytoplasm of the combined drug group showed shrinkage and vacuole, and many cells ruptured. The results of MTT colorimetric assay showed that compared with the control group, the ursolic acid group treated with SW480 cells showed obvious inhibition of cells (P<0.05), and the oxaliplatin group showed obvious cell inhibition after treatment for 24 hours. (P<0.05), the inhibition was significantly higher after 48h compared with the control group (P<0.01), and the combination group showed extremely significant cell inhibition after treatment for 24h (P<0.01). The results of flow cytometry showed that compared with the control group, the percentage and apoptosis rate of G1/G0 phase cells in other groups were significantly increased (P<0.05), and the percentage of S phase and G2/M phase were significantly reduced (P<0.05).Western blot results showed that compared with the control group, the expression of Bax protein and Bcl-2 protein in tumor cells of other drug treatment groups was significantly increased and significantly decreased (P<0.05).*

Conclusion: *Ursolic acid combined with oxaliplatin can significantly inhibit the proliferation of rectal cancer cells, induce cell cycle arrest and promote apoptosis, and the mechanism may be related to the regulation of Bcl-2 and Bax protein expression.*

Keywords: *Ursolic acid, Oxaliplatin, Rectal cancer, Proliferation, Mechanism of action.*

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Introduction

Rectal cancer is one of the common malignant tumors of the digestive tract, often occurring in the dentate line to the rectosigmoid colon. Insidious early onset, rapid disease progression and high malignancy make diagnosis difficult to find, so the prognosis is poor. In recent years, with the development of population aging in China, the incidence rate of colon cancer is on the rise, and there are about 100 new cases every year in the global scope, seriously threatening the health and quality of life of patients[(1-2)].

So far, radical resection of rectal cancer is still the only hope for the cure of rectal cancer patients. However, due to the complex relationship of rectal cancer resection and its deep location in the pelvic cavity, the operation is often not clear and thorough. Most patients have a recurrence in about two years, and the reason for recurrence is mainly due to the undifferentiated microscopic cancer cells not completely cleared during the operation[(3)].

The combination of postoperative chemotherapy has become an effective method for the treatment of postoperative rectal cancer, but due to the presence of drug toxicity and drug resistance in some patients, the chemotherapy effect or prognosis of rectal cancer patients is seriously affected. Therefore, it has become the focus of clinical scholars to explore the combination of drugs to enhance the efficacy and reduce adverse reactions at the same

time. Oxaliplatin is a third-generation platinum compound, which is often used as an anti-tumor chemotherapy drug in clinic. Due to other platinum drugs in terms of efficacy and drug dynamics, oxaliplatin has obvious toxicity to nerves and liver[4-5].

Arbutin is a pentacyclic triterpene compound, also known as ursolic acid, which is widely found in natural plants and herbs such as bearberry and Hedyotis diffusa. In recent years, its anti-inflammatory, anti-infective and hepatoprotective effects, especially its anti-tumor effects, have attracted much clinical attention, but few reports on its specific mechanism of action[6]. Therefore, in this study, ursolic acid combined with oxaliplatin was used to treat human rectal cancer cells, aiming to investigate the effect of the combination of two drugs on cell proliferation and apoptosis and the mechanism of action.

Materials and methods

Laboratory reagents and instruments

Human rectal cancer cell line SW480 was provided by the Chinese academy of medical sciences. Ursolic acid was provided by Beijing institute of pharmaceutical and biological products; Oxaliplatin injection was provided by shenzhen haiwang pharmaceutical co., LTD. RPMI1640 medium powder and fetal bovine serum were provided by Gibo BRL company, USA. MTT reagent, trypsin and protein extraction kit were provided by biyuntian institute of technology. Mouse monoclonal antibodies against human Bax and Bcl-2 were provided by Santa Cruz, USA.

The ultra-clean workbench (SW-CJ-2FD) was provided by suzhou antai air technology co., LTD. Inverted microscope provided by Garl Zeiss, Germany; Supercentrifuge (CP100WX) supplied by Hitachi, Japan; Fully automatic SpectraMax M2e was provided by molecular instruments, inc. Carbon dioxide incubator (3111) supplied by semel fisher, USA; Flow cytometry (FACSCalibur) was provided by B-D corporation, USA.

Cell culture and grouping

Human rectal cancer cell line SW480 was cultured in RPMI-1640 medium containing 10% fetal bovine serum at 37oC and 5% CO2. The liquid was changed every day, and the logarithmic growth phase cells were selected for the experiment.

All cells were divided into control group (with culture solution only), ursolic acid group (45 μmol/L ursolic acid treatment), oxaliplatin group (10mg/L oxaliplatin treatment), and combination group (45 μmol/L ursolic acid +10mg/L oxaliplatin).

Experimental methods

• Observation of cell morphology: human rectal cancer cell line SW480 was cultured for 24h at logarithmic growth stage, adding 45 μmol/L ursolic acid, 10mg/L oxaliplatin and 45 μmol/L ursolic acid +10mg/L oxaliplatin, respectively, and observing the changes of cell morphology after 72h under an inverted microscope. RPMI1640 culture medium was used as the blank control group.

• MTT colorimetry was used to detect the changes of cell proliferation: human rectal cancer cell line SW480 cells at logarithmic growth stage were inoculated on a 96-well culture plate and cultured in a 50 mL/CO2 incubator at 37oC for 24h, 100 μl per well. 45 μmol/L ursolic acid, 10 mg/L oxaliplatin and 45 μmol/L ursolic acid +10 mg/L oxaliplatin were added to culture medium and cultured for 48h, each group has 5 compound holes. Before the end of culture, 500 μg/mL MTT was added to avoid light and incubated at 37 oC for 4h. After cultivation, the supernatant was discarded. DMSO150 μL/ well was added to each well and shaken for 10min in dark to dissolve the crystallites. Cell proliferation inhibition rate (%) =1- OD value in the experimental group/ OD value in the control group.

• Cell cycle changes: human rectal cancer cell line SW480 was cultured for 24h with 45 μmol/L ursolic acid, 10 mg/L oxaliplatin and 45 μmol/L ursolic acid +10 mg/L oxaliplatin, and cultured normal saline was used as the reference control group. After 48h, the cells were collected and the changes in cell cycle and apoptosis rate were detected by flow cytometry.

• Protein expressions of Bax and Bcl-2 in tumor cells: 3×106 cells in each group treated with drugs for 24h were collected, and the protein expressions of Bax and Bcl-2 were detected by Western blot.

Statistical methods

The data of this study were analyzed by SPSS20.0 software package. The measurement data were expressed by mean ± standard deviation ($\bar{x} \pm s$). The comparison between groups was analyzed by one-way ANOVA. The count data were analyzed by chi-square test. $P<0.05$ was considered as statistical significant difference.

Results

The inhibition of morphological changes of SW480 cells in vitro by each group of drugs

Observation under inverted microscope showed that the cells in the control group showed good logarithm growth on the wall surface; The growth of the ursolic acid group and the oxaliplatin group decreased, the morphology became round and the growth was inhibited; The cytoplasm of the combined drug group showed shrinkage and vacuole, and many cells ruptured. As shown in figure 1.

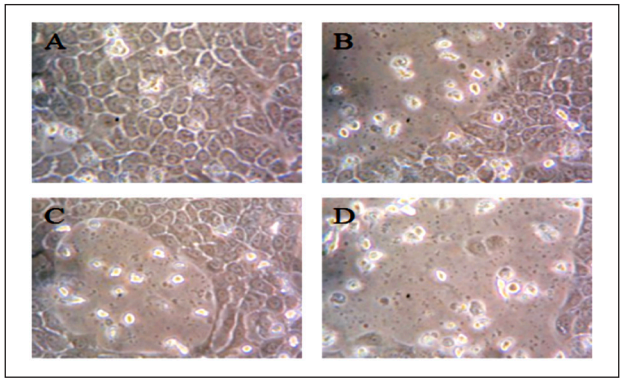

Fig. 1: Morphological changes of SW480 cells in vitro were inhibited by drugs in each group.
A: *control group,* ***B:*** *ursolic acid group,* ***C:*** *oxaliplatin group,* ***D:*** *combination group.*

Effects of each group on the proliferation of SW480 cells

The results of MTT colorimetric assay showed that compared with the control group, the ursolic acid group treated with SW480 cells showed obvious inhibition of cells (P<0.05), and the oxaliplatin group showed obvious cell inhibition after treatment for 24 hours. (P<0.05), the inhibition was significantly higher after 48h compared with the control group (P<0.01), After 24h of treatment, the combination group showed extremely significant cell inhibition (P<0.01), which was statistically significant different from the ursoic acid group and oxaliplatin group (P<0.05). See table 1.

Group	SW480 cells		
	24h	48h	72h
Control	0.581±0.117	0.956±0.401	1.236±0.059
Ursolic acid (45μmol/L)	0.572±0.034	0.743±0.067*	1.034±0.045*
Oxaliplatin (10mg/L)	0.460±0.036*	0.568±0.003**	0.649±0.049**
Combination (45μmol/L ursolic acid +10mg/L oxaliplatin)	0.305±0.108**	0.412±0.063**	0.421±0.049**

Table 1: Effects of each group on the proliferation of SW480 cells ($\bar{x}\pm s$).
*Note: *means P<0.05 compared with the control group; **P<0.01.*

Effects of each group on cell cycle and apoptosis rate of SW480 cells

The results of flow cytometry showed that, compared with the control group, the percentage and apoptosis rate of G1/G0 phase cells in other groups were significantly increased (P<0.05), and the percentage of S phase and G2/M phase were significantly reduced, with statistically significant differences (P<0.05). See table 2.

Group	G1/G0	S	G2/M	Apoptosis rate
Control	36.45±3.44	41.09±3.81	20.19±0.62	1.58±0.36
Ursolic acid (45μmol/L)	40.15±2.74*	36.34±2.67*	16.57±4.25*	20.10±2.40*
Oxaliplatin (10mg/L)	45.27±3.10*	34.68±1.07*	12.47±3.78*	25.19±1.36*
Combination (45μmol/L ursolic acid +10mg/L oxaliplatin)	60.18±5.02*	31.14±1.99*	6.58±3.14*	35.19±2.37*

Table 2: Effect of each group on cell cycle of SW480 cells (%).
*Note: *means P<0.05 compared with the control group.*

Effects of each group on Bax and Bcl-2 protein expression in SW480 cells

Western blot results showed that, compared with the control group, Bax protein expression in tumor cells in other drug treatment groups was significantly increased, and Bcl-2 protein expression was significantly decreased (P<0.05), as shown in figure 2.

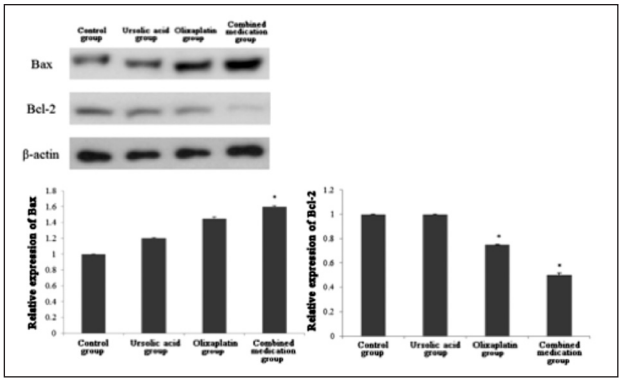

Fig. 2: Effects of each group on Bax and Bcl-2 protein expression in SW480 cells
*Note: *means P<0.05 compared with the control group*

Discussion

In recent years, the incidence and fatality rate of rectal cancer have been increasing year by year in China, and some patients were already in the middle and late stage of tumor tissue when they were seen for treatment and had distant metastasis[7]. At present, surgical resection is still the main method for clinical treatment of rectal cancer, but the tumor recurrence rate and metastasis rate are higher after surgery. Especially when the tumor breaks through

the serosa layer, the cancer cells are easily implanted into the serosal surface of the peritoneum or other organs. In the middle of the operation, lymph nodes were swept away from cancer cells, and the immune function of the patients decreased after surgery, which in turn increased the recurrence and metastasis of postoperative cancer cells[8-9]. Many studies have shown that[10-11], tumor development and cell proliferation are related to the destruction of the dynamic balance of apoptosis.

The relationship between apoptosis and tumor has become a hot topic in recent years, and the Bcl-2 family has received much attention. Bax and Bcl-2 belong to the pre-apoptotic gene and anti-apoptotic gene in the Bcl-2 family, respectively. Bax is mainly distributed in epithelial cells close to the surface, and is an important mediator of p53-dependent apoptosis signaling pathway, playing an important role in promoting apoptosis. Bcl-2 is the earliest apoptosis-related gene, which can inhibit apoptosis and shorten the cell cycle, thereby promoting the growth of cancer cells[12].

As a secondary metabolite, ursolic acid has been proved to have great medicinal value in the prevention and treatment of tumors in recent years[13-14]:

Ursolic acid can regulate the metabolism of cancer cells, activate the pathway leading to cell death, inhibit the metastasis of cancer cells; Ursolic acid can exert anti-cancer effect by inhibiting MAPK/ERK, P13K/AKT/mTOR and other signaling pathways; On the effect of apoptosis regulatory protein, ursolic acid can change the inhibition of anti-apoptotic protein bcl-2 and promote the enhancement of Bax activity; The effect on tumor angiogenesis and metastasis; Ursolic acid has been confirmed to significantly inhibit COX-2 activity, thereby inhibiting the progression of inflammation.

Oxaliplatin, like other platinum drugs, is cytotoxic almost by destroying DNA, thereby stopping the replication of cancer cells and leading to apoptosis. Oxaliplatin has achieved certain efficacy in anticancer treatment, but its side effects and dose limits make the clinical efficacy limited[15]. In this study, ursolic acid combined with oxaliplatin was used to treat human rectal cancer cells. The results of MTT colorimetric assay showed that compared with the control group, the ursolic acid group treated with SW480 cells showed obvious cytostatic effect ($P<0.05$), oxaliplatin group showed obvious cell inhibition after treatment for 24h ($P<0.05$). After 48h, the inhibition was significantly higher than that of the control group ($P<0.01$).

The results of flow cytometry showed that compared with the control group, the percentage of G1/G0 phase and apoptosis rate of other groups were significantly increased ($P<0.05$), and the percentages of S phase and G2/M phase were significantly decreased ($P<0.05$). This suggests that ursolic acid has a significant inhibitory effect on the proliferation of rectal cancer cells. Compared with ursolic acid or oxaliplatin alone, the combined treatment group had a very significant effect on inhibiting the proliferation of cancer cells and promoted apoptosis by inducing cell cycle arrest.

In order to further explore possible mechanisms of ursolic acid on colorectal cancer cell proliferation inhibition, this study by Western blot method test results showed that compared with the control group, other drug treatment group Bax protein expression in tumor cells increased significantly, the Bcl-2 protein expression decreased significantly ($P<0.05$), suggesting the mechanism of action of ursolic acid inhibit colorectal cancer cell growth may be by adjusting the Bax, Bcl-2 protein levels.

In summary, ursolic acid combined with oxaliplatin can significantly inhibit the proliferation of rectal cancer cells, induce cell cycle arrest and promote apoptosis, and the mechanism may be related to the regulation of Bcl-2 and Bax protein expression.

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