

ORIGINAL ARTICLE

Inhibitory effect of 5-Fluorouracil on the proliferation of human osteosarcoma cells *in vitro*

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Summary

Purpose: To evaluate the inhibitory effect of 5-fluorouracil (5-FU) in combination with cisplatin on the proliferation of human osteosarcoma cells *in vitro*.

Methods: Three groups of human osteosarcoma U2OS cells were cultured in DMEM medium supplemented with the following drugs: 20 µg/mL 5-FU, 50 µg/mL 5-FU and 50 µg/mL 5-FU in combination with 0.5 mg/L cisplatin. After culture at 24, 48 and 72 h, the inhibition of proliferation rate of U2OS cells was calculated by CCK-8 cell kit. Cell invasiveness was assessed by Transwell assay. Flow cytometry was used for monitoring the cell apoptosis.

Results: 5-FU inhibited the growth of osteosarcoma cells, and the results of different concentrations of 5-FU were significantly different. The growth of U2OS cells decreased significantly within 24-72 h, and the concentration of 5-FU increased with time. The inhibition of the shift was more obvious, and the combined drug inhibition was significantly higher than the 20µg/ml 5-FU Group, 0.5mg/L Cisplatin Group and 50µg/ml 5-FU Group. After 72 h, the mean inhibitory rates of 20 µg/mL 5-FU, 50 µg/mL 5-FU, 50 µg/ml 5-FU in combination with 0.5 mg/L cisplatin, and 0.5 mg/L cisplatin were 12.54±1.26%, 22.17±0.59%, 32.54±1.25%, 20.84±0.83% respectively, and the difference was signifi-

cant ($p < 0.05$). Results of cell invasion assay showed that after culturing for 48 h, the mean number of cells penetrating the membrane was 22.84±5.27 in the culture group of 0.5 mg/L cisplatin, 30.57±5.68 in the culture group of 20 µg/mL 5-FU, 18.68±4.88 in the culture group of 50 µg/mL 5-FU, and 9.84±3.64 in the culture group of 50 µg/mL 5-FU in combination with 0.5 mg/L cisplatin, respectively, and 72.00±7.52 in the control group, showing statistical differences in each group ($p < 0.05$). The apoptosis of the control group was significantly lower than that of the other groups ($p < 0.05$). Apoptosis rate of the 20µg/mL 5-FU group was significantly lower than that of the 0.5mg/L cisplatin group, the 50µg/mL 5-FU group and the 50 µg/ml 5-FU group in combination with 0.5mg/L cisplatin ($p < 0.05$). There was no difference in apoptosis between 0.5mg/L cisplatin and 50µg/mL 5-FU group ($p > 0.05$).

Conclusion: 5-FU in combination with cisplatin exerts an inhibitory effect on the proliferation and invasion of human osteosarcoma cells *in vitro*, and can promote cell apoptosis.

Key words: 5-fluorouracil, human osteosarcoma cell, cell proliferation, proliferation inhibition rate, cell culture *in vitro*

Introduction

Osteosarcoma (OS) originates from mesenchymal tissues. Its incidence accounts for 0.2% of all human malignancies, and it often occurs in adolescents between 10 and 20 years old. It has been

reported that the incidence of OS in adolescents accounted for 70% of all the patients with OS. The onset of OS primarily occurs in the rapidly growing metaphyseal regions. Due to its rapid growth,

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lung metastases are very likely to occur in the early stage of disease [1,2]. At present, treatment of OS involves mainly surgery and neoadjuvant chemotherapy, as well as other comprehensive treatments. These treatments effectively extended the OS patient survival with the 5-year survival rate being increased from 10-20% to the current 70%. However, emergence of multidrug resistance in patients due to long-term use of drugs seriously affected the survival of patients with OS, and also offset clinical efficacy of the drugs. Statistical analyses showed that death due to recurrence of OS accounted for 70% [3-5]. Multiple factors can contribute to drug resistance in patients with OS. It has been reported that apoptotic factors were closely related to strengthened DNA repair [6]. Therefore, developing innovative approaches to combat drug resistance is one of the keys in the clinical treatment of OS.

Cisplatin is an important chemotherapeutic drug for the treatment of OS in the clinic. Studies have shown that the survival of patients with OS is significantly improved after treatment with cisplatin. 5-Fluorouracil (5-FU) has been used clinically as a metabolic anticancer drug for many years, mainly in gastric cancer, liver cancer, ovarian cancer and colon cancer etc. It is still the drug of choice for chemotherapy in the treatment of gastrointestinal cancers [7]. *In vivo*, 5-FU is converted into the active metabolite 5F-dUMP (5-fluorouracil deoxyribonucleic acid) through ribosylation and phosphorylation, resulting in inhibition of thymidylate synthase, the enzyme that catalyzes the conversion of deoxyuridine to deoxythymidylate in a reductive methylation. Deoxythymidylate is one of the building blocks of DNA replication. Thus, depletion of deoxythymidylate has serious consequences for DNA synthesis and repair, ultimately leading to apoptosis and cell cycle arrest [8,9]. Recent studies have shown that 5-FU in combination with cisplatin has achieved good results in the treatment of lung cancer and gastric cancer, but there is no clear report on whether OS can be treated this way. In this study, the inhibitory effect of 5-FU at different concentrations and in combination with cisplatin was explored on OS cell proliferation.

Methods

Materials and instruments

The following materials were purchased from respective sources: human osteosarcoma cell line U2OS from Shanghai Xin Yu Biotechnology Co., Ltd., China; 0.25% trypsin, fetal bovine serum (FBS), MTT, DMEM media, and 5-FU from Sigma, USA; cisplatin from Simeiquan Biotechnology Co., Ltd., China; CCK-8 kit from

Beyotime Biotechnology Co., Ltd., China; Transwell cell culture chambers from Corning, USA. A DxFLEX flow cytometer from Beckman Coulter was used in this study.

Cell culture

The human OS cell line U2OS was cultured in DMEM supplemented with 10% FBS and a dual antibiotic solution containing penicillin (100 U/mL) and streptomycin (10 µg/mL). The incubation was maintained at 37°C in a humidified atmosphere containing 5% CO₂. The DMEM medium was changed every 24 h. Cells were passaged following digestion with 0.25% trypsin (containing EDTA). The cells in logarithmic growth phase were harvested for the following experiments.

Cell viability assay

The cells in logarithmic growth phase (80%) were digested with 0.25% trypsin and counted. After washing with phosphate buffered saline (PBS) and resuspension, the cells were adjusted to a concentration of 1×10^5 cells/mL and seeded into 96-well plates (100 µL/well). When the cells were attached to the bottom of the well and started to grow after culturing for 24 h, the following reagents were added respectively into triplicate wells: 0.5 mg/L cisplatin, 20 µg/mL 5-FU, 50 µg/mL 5-FU, and 50 µg/mL 5-FU in combination with 0.5 mg/L cisplatin. Cell viability assay was performed by using the CCK-8 kit after culturing for 24, 48 and 72 h. Absorbance value (A value) of each well was obtained at 450 nm by using a microplate reader. This experiment was carried out 3 times.

Apoptosis test

Cells (5 mL) at a concentration of 1×10^5 cells per mL were seeded into T25 flasks. In these flasks were added the same volume of serum, followed by addition of 0.5 mg/L cisplatin, 20 µg/mL 5-FU, 50 µg/mL 5-FU, and 50 µg/mL 5-FU in combination with 0.5 mg/L cisplatin, respectively. After culturing for 24 h, the media were aspirated from each flask and saved for reuse. The cells were washed with PBS, and then digested with 0.25% trypsin, allowing detachment from the flask bottom. The media containing reagents were re-added into the culture flasks and mixed well to stop trypsin digestion. The final cell suspension was centrifuged at 1000 rpm for 10 min. After the supernatant was discarded, the cells were washed with PBS, followed by another centrifugation at 1000 rpm for 10 min. 70% ethanol chilled at -20°C was added to the cells. After the mixture was mixed by blowing in air bubbles, cell fixation was done by incubation at 4°C for 90-120 min. Following removal of the fixation agent, the fixed cells were re-suspended in PBS and filtered through a 300-mesh filter, followed by centrifugation at 1000 rpm for 10 min. Propidium iodide (PI) staining solution was added to the fixed cells, followed by incubation for 30 min in the dark. Flow cytometry was performed and the data were processed by using the software Cell Quest.

Cell invasion assay

To each Transwell chamber 40 µL of pre-chilled and diluted Matrigel solution (500 µg/mL) were added.

The plate with the chambers was incubated at 37°C for 6 h. Then, the remaining Matrigel solution was aspirated by using a pipette, followed by addition of DMEM (serum-free) and incubation at 37°C for 30 min. The coated invasion chambers were then ready for use. Cells were prepared and treated with 5-FU in the same way as described in the section of Cell Viability Assay. After the five groups of cells (including the control group, 0.5 mg/L cisplatin, 20 µg/mL 5-FU, 50 µg/mL 5-FU, and 50 µg/mL 5-FU in combination with 0.5 mg/L cisplatin) were cultured for 48 h, 0.25% trypsin was added to detach the cells, followed by addition of DMEM (serum-free) to adjust the cell concentration to 1×10^6 cells per ml. To the upper compartment of triplicate Transwell chambers 200 µL of the cell suspension from each group was added, and to the lower compartment 600 µL of DMEM supplemented with 10% FBS was added. The plate with the chambers was incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 48 h. A cotton swab was used to gently rub the area in the upper compartment to remove the non-invading cells. The cells in the lower compartment were fixed at room temperature for 20 min, followed by hematoxylin and eosin (HE) staining. The invading cells were observed under a microscope and counted in several fields.

Statistics

SPSS 20.0 statistics software (Shanghai Cabit Information Technology Co., Ltd.) was used to process all the data. Measurement data were expressed as mean \pm standard deviation (\pm s). Comparisons between groups were performed by one-way analysis of variance (ANO-

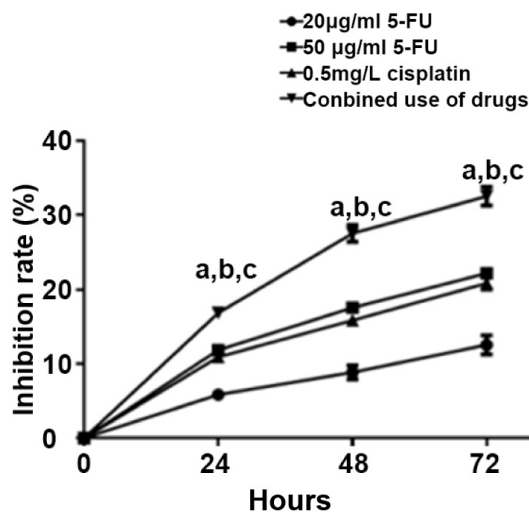


Figure 1. Cell growth. 5-FU and 0.5 mg/L cisplatin inhibited the growth of OS cells, and the results of different concentrations of 5-FU were significantly different. The growth ability of U2OS cells decreased significantly within 24-72 h, and the concentration of 5-FU increased with time. The inhibition of the shift was more obvious, and the combined drug inhibition was significantly higher than the other three groups. ^a $p < 0.05$, compared with the culture group of 0.5 mg/L cisplatin; ^b $p < 0.05$, compared with the culture group of 20 µg/mL 5-FU; ^c $p < 0.05$, compared with the culture group of 50 µg/mL 5-FU.

VA), indicated by F, and LSD-t test was used for pairwise comparisons within the group. The difference was statistically significant if $p < 0.05$.

Results

Effect of 5-FU on proliferation of OS U2OS cells

Cell viability assay using CCK-8 showed that 5-FU inhibited the growth of OS cells, and variable outcomes were observed at different drug concentrations. The U2OS cell viability decreased significantly after culturing with 5-FU for 24-72 h. The inhibitory effect of 5-FU was greater over time and when the drug concentration was increased. In addition, the combination therapy showed higher inhibition than the other three groups. After culturing with 5-FU for 72 h, the cell proliferation inhibition rates were $12.54 \pm 1.26\%$, $22.17 \pm 0.59\%$, $32.54 \pm 1.25\%$ and $20.84 \pm 0.83\%$ respectively, in the culture groups of 20 µg/mL 5-FU, 50 µg/mL 5-FU, 50 µg/mL 5-FU in combination with 0.5 mg/L cisplatin and 0.5 mg/L cisplatin. The differences between them were statistically significant ($p < 0.05$). The results are shown in Table 1 and Figure 1.

Cell invasiveness

The U2OS cell invasiveness after treatment with different concentrations of 5-FU was determined by using Transwell assay. After treatment for 48 h, the number of cells penetrating the membrane was 22.84 ± 5.27 in the culture group of 0.5 mg/L cisplatin, 30.57 ± 5.68 in the culture group of 20 µg/mL 5-FU, 18.68 ± 4.88 in the culture group of 50 µg/mL 5-FU, and 9.84 ± 3.64 in the culture group of 50 µg/mL 5-FU in combination with 0.5 mg/L cisplatin, respectively. Compared with 72.00 ± 7.52 in the control group, the other groups were significantly reduced ($p < 0.05$). The number of cells penetrating the membrane in 50 µg/ml 5-FU in combination with 0.5 mg/L cisplatin was significantly lower than those in 0.5 mg/L cisplatin group, 20 µg/mL 5-FU group and 50 µg/mL 5-FU group

Table 1. Effect of 5-FU at different concentrations on the proliferation of OS U2OS cells (mean \pm SD)

Group	Inhibition rate
0.5 mg/L cisplatin	20.84 ± 0.83
20 µg/mL 5-FU	12.54 ± 1.26^a
50 µg/mL 5-FU	22.17 ± 0.59^b
50 µg/mL 5-FU in combination with 0.5 mg/L cisplatin	32.54 ± 1.25^{abc}

^a $p < 0.05$, compared with the culture group of 0.5 mg/L cisplatin;

^b $p < 0.05$, compared with the culture group of 20 µg/mL 5-FU;

^c $p < 0.05$, compared with the culture group of 50 µg/mL 5-FU.

($p < 0.05$). Compared with the 20 $\mu\text{g}/\text{mL}$ group, 50 $\mu\text{g}/\text{mL}$ group was reduced significantly ($p < 0.05$). The results are shown in Figure 2.

Cell apoptosis analysis by flow cytometry

Flow cytometry showed that 0.5 mg/L cisplatin, 5-FU monotherapy at different concentrations (20 and 50 $\mu\text{g}/\text{mL}$) and 5-FU combination therapy with cisplatin (50 $\mu\text{g}/\text{mL}$ 5-FU in combination with 0.5 mg/L cisplatin) all had a significant difference in cell apoptosis ($p < 0.05$). The apoptosis of the control group was significantly lower than that of the other groups ($p < 0.05$). The apoptosis rate of 20 $\mu\text{g}/\text{mL}$ 5-FU group was significantly lower than that of 0.5 mg/L cisplatin group, 50 $\mu\text{g}/\text{mL}$ 5-FU group and 50 $\mu\text{g}/\text{mL}$ 5-FU in combination with 0.5 mg/L

cisplatin ($p < 0.05$). There was no difference in apoptosis between the 0.5 mg/L cisplatin group and 50 $\mu\text{g}/\text{mL}$ 5-FU ($p > 0.05$). The results are shown in Table 2.

Discussion

Incidence of OS is the highest among tumors occurring in the adolescents. Studies suggested that OS as a differentiated disease occurs due to abnormalities in the differentiation of bone marrow mesenchymal stem cells into osteoblasts. It often happens in adolescents when bone is growing fast [10]. 5-FU is the most commonly used pyrimidine anticancer drug in the clinic, and it has occupied a very important place among various agents for cancer chemotherapy since its discovery. It was widely used to treat various malignancies such as pancreatic cancer, gastric cancer and breast cancer with excellent efficacy [11-13]. As a cell cycle-specific agent, 5-FU can be metabolized to 5-fluorouracil deoxyribonucleic acid, which blocks the action of thymidylate synthase, thus stopping the production of RNA and DNA [14]. The metabolic pathways of tumor tissues are basically the same as that of normal tissues, thus allowing the inhibition of tumor progression by using 5-FU. However, long-term use of 5-FU at high doses will result in decreased drug sensitivity and drug resistance, which is one of the reasons leading to failure of chemotherapy in the late stage [15]. Cisplatin is a cell cycle nonspecific anticancer drug. It can inhibit the replication of DNA in tumor cells, resulting in destruction of the cell membrane structure. Cisplatin was widely used in clinical chemotherapy of various malignancies including ovarian cancer, lung cancer, esophageal cancer, head and neck squamous cell carcinoma, thyroid cancer and osteosarcoma [16,17].

In a study by Shinoda et al. it was shown that low-dose cisplatin in combination with 5-FU can effectively improve the median survival time in patients with esophageal cancer [18]. So far there has been no report on combination therapy of 5-FU

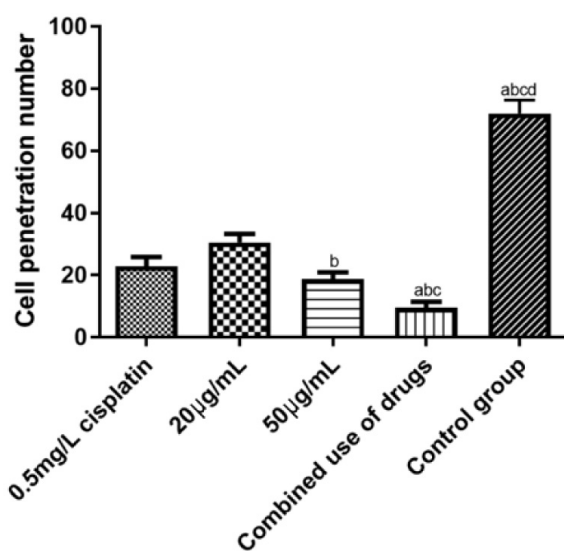


Figure 2. Cell invasion using Transwell assay. Compared with the control group, invasion of the other groups was significantly reduced ($p < 0.05$). Invasion of 50 $\mu\text{g}/\text{mL}$ 5-FU in combination with 0.5mg/L cisplatin group was reduced, and was significantly different from 0.5mg/L cisplatin group, 20 $\mu\text{g}/\text{mL}$ and 50 $\mu\text{g}/\text{mL}$ group ($p < 0.05$). Compared with 20 $\mu\text{g}/\text{mL}$ group, 50 $\mu\text{g}/\text{mL}$ group was reduced significantly ($p < 0.05$). ^a $p < 0.05$, compared with the control group; ^b $p < 0.05$, compared with 0.5 mg/L cisplatin group; ^c $p < 0.05$, compared with 20 $\mu\text{g}/\text{mL}$ 5-FU; ^d $p < 0.05$, compared with 50 $\mu\text{g}/\text{mL}$ 5-FU.

Table 2. Cell apoptosis in the different groups

Group	Apoptosis rate	F	p
Control group	4.25±0.84		
0.5 mg/L cisplatin	21.84±3.74 ^a		
20 $\mu\text{g}/\text{mL}$ 5-FU	15.22±2.15 ^{ab}	29.061	<0.001
50 $\mu\text{g}/\text{mL}$ 5-FU	22.95±3.81 ^{ac}		
50 $\mu\text{g}/\text{mL}$ 5-FU in combination with 0.5 mg/L cisplatin	35.25±5.62 ^{abcd}		

^a $p < 0.05$, compared with the control group; ^b $p < 0.05$, compared with 0.5 mg/L cisplatin group; ^c $p < 0.05$, compared with 20 $\mu\text{g}/\text{mL}$ 5-FU; ^d $p < 0.05$, compared with 50 $\mu\text{g}/\text{mL}$ 5-FU.

and cisplatin in OS treatment. In this study, the inhibitory effect of 5-FU at different concentrations and in combination with cisplatin was explored on osteosarcoma U2OS cell proliferation. It was found that the inhibition of cell proliferation rate in the culture group of 20 µg/mL 5-FU was significantly lower than that in the culture group of 50 µg/mL 5-FU. This indicated that the inhibition rate increased over time and along with the drug concentration. In the case of combination therapy of 5-FU and cisplatin, the inhibition rate was even higher than those in the three monotherapy groups. Thus, in terms of cell proliferation inhibition rate, higher 5-FU concentration and combination therapy gave better inhibitory effects. Through cell invasion assay, it was found that the cell invasiveness decreased along with the increase of 5-FU concentration. In the case of combination therapy of 5-FU and cisplatin, the decrease of cell invasiveness was even higher than those in the two monotherapy groups. The above findings suggested that the combination therapy was more ef-

ficient than monotherapy in the treatment of OS. This is in accordance with a finding in literature that combination therapy can further reduce the incidence of adverse reactions and complications [19]. Apoptosis is an important means of clinical treatment of tumors [20]. In this study, we finally found that apoptosis of different groups of 5-FU and 0.5mg/L cisplatin can promote tumor apoptosis. 50µg/ml 5-FU in combination with 0.5mg/L cisplatin is superior to other groups in promoting tumor cell apoptosis, which indicates that 50µg/ml 5-FU in combination with 0.5mg/L cisplatin can effectively promote the apoptosis of OS U2OS cells to inhibit tumor growth.

In summary, 5-FU in combination with cisplatin exerted an inhibitory effect on the proliferation and invasion of human OS cells *in vitro*, and can promote the cell apoptosis.

Conflict of interests

The authors declare no conflict of interests.

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