

The Effect of Fullerenol Combined with Cisplatin on the Proliferation of Cervical Cancer HeLa Cells

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Abstract

This study aimed to investigate the effect of fullerenol combined with cisplatin on the proliferation of cervical cancer HeLa cells in order to provide new ideas and laboratory theoretical basis for the clinical treatment of cervical cancer. Cervical cancer cell line HeLa cells *in vitro* were treated with different concentrations of fullerenol, different concentrations of cisplatin, and different concentrations of fullerenol combined with cisplatin, after 24 h, 48 h, 72 h, microscope changes in cell morphology; MTT assay was used to determine the effect of drugs on the proliferation of HeLa cells. Fullerenol and cisplatin alone can inhibit the proliferation of HeLa cells in a dose-dependent and time-dependent manner; compared with cisplatin alone, different concentrations of fullerenol combined with cisplatin significantly increased the apoptosis rate of HeLa cells ($P < 0.05$). The inhibition of fullerenol combined with cisplatin on HeLa cells *in vitro* is more significant, resulting in a stronger anti-cancer effect.

Keywords

Fullerenol, Cisplatin, HeLa Cells, Proliferation, Apoptosis

1. Introduction

Cervical cancer is one of the most common female cancers; female cancer mortality rate was 3.28/100,000. In China, about 2 - 3 million women were died of cervical cancer every year; the incidence rate of cervical cancer

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in young women is increasing [1] [2]. Surgery and radiotherapy are the main treatments for cervical cancer; in the early stage of the disease surgical treatment timely is taken, and in most advanced stage radiotherapy is taken. Application of chemotherapy in cervical cancer at home and abroad has carried out basic and clinical research in recent years and achieved satisfactory results, which result in improving 5-year survival rate and determining the status of chemotherapy in the treatment of cervical cancer [3]-[7]. However, the disadvantages of chemotherapy drugs are poor specificity and easy resistance; in response to these shortcomings, fullereneol may provide a new method to improve the problem. Fullereneol is a water-soluble fullerene derivative by introducing 18 - 20 hydroxy group at fullerene carbon, has a variety of biological activities, and can cause cell autophagy and induce apoptosis effect characterized by a dose-dependent and time-dependent relationship [8]; in addition, it can clear free radicals of blood and superoxide radicals generated by xanthine and xanthine oxidase in aqueous solution [9]. In this regard, fullereneol has a higher biomedical value worth exploring. This study aimed to explore the possibility of producing safer and more effective anti-tumor effect with fullereneol combined with cisplatin by observing the changes of fullereneol, cisplatin and fullereneol combined with cisplatin on HeLa cells proliferation and comparing different fullereneol cisplatin and fullereneol cisplatin monotherapy effect, which provided experimental evidence and theoretical basis by using fullereneol in the treatment of cervical cancer.

2. Materials and Methods

2.1. Drugs and Reagents

Fullereneol (supplied by Professor Dianbao Chen, Qingdao University of Science and Technology), cisplatin (Jiangsu Stockhausen Pharmaceutical Co., Ltd.), dimethyl sulfoxide (DMSO, Sigma Company), MTT (MTT) was purchased from Haibi sky Biotechnology Co., Ltd., CO₂ incubator, automatic microplate reader.

2.2. Cell Culture and Subculture

Cervical cancer cell line HeLa cells (Shanghai Cell Bank), culture medium containing 10% fetal calf serum and 0.05 g/L penicillin and 0.05 g/L streptomycin and RPMI1640 medium (Gibco Company); containing 5% CO₂, 37°C saturated humidity incubator; grown for 2 - 3 days; 0.25% trypsin digestion and passage. Logarithmic growing cells for experiments.

2.3. Fullereneol Formulated Solution

Different concentrations of fullereneol were treated with DMEM medium without fetal calf serum, then filter sterilization by 0.22 mm microporous membrane when it will be used.

2.4. Inverted Microscope to Observe the HeLa Cell Morphological Changes

Logarithmic growth phase of HeLa cells after counting to 10⁵/mL concentration seeded in culture flasks of 25 cm² bottle 2 mL, after being adherent cells to be administrated drugs, fullereneol group (concentrations of 2.5, 5, 10 ug/mL), cisplatin (1, 2.5, 5, 10 ug/mL), the combined group (5 ug/mL cisplatin + 2.5, 5, 10 ug/mL fullereneol), after 24, 48, 72 hours, they were observed the cell morphology by using inverted microscope.

2.5. MTT Cell Viability Assay

Experimental HeLa cells were divided into four groups: control group (Only add fresh medium); fullereneol group (concentration, respectively 2.5 ug/mL, 5 ug/mL, 10 ug/mL); cisplatin (1 ug/mL, 2.5 ug/mL, 5 ug/mL, 10 ug/mL); the combined group (5 ug/mL cisplatin + 2.5 ug/mL, 5 ug/mL, 10 ug/mL fullereneol). Each group provided three holes, the drug concentration is diluted by medium to experiment with various concentrations. Making HeLa cells in logarithmic phase into single cell suspension adjust the cell concentration of 2×10^4 /mL, which were seeded in 96-well culture plate and set 37.0°C, 5% CO₂ culture box to culture for 24 hours. The experimental groups were added with different concentrations of cisplatin and fullereneol making the final concentration meet the above requirements, while the control group was added with equal volume of culture medium. After 24, 48, 72 hours, the plates were scheduled for the detection of each well 20 μ l MTT (5 mg/mL) solution in CO₂ incubator incubated for 4 h, then the supernatant was discarded, trace shaker shaken for 15 minutes after 150 μ l DMSO each well, the absorbance was measured OD values of each well using a microplate reader at 490

nm finally. The experiment was repeated three times. Calculated growth inhibition rate (IR) of HeLa cells according to the following formula. $IR = (1 - \text{average OD value of experimental group} / \text{average OD value of control group}) \times 100\%$

2.6. Statistical Analysis

All statistical analyses were performed using SPSS software (version 17.0, SPSS Inc., USA). Samples were compared using the t test. Significance level $\alpha = 0.05$. $P < 0.05$ was considered statistically significant.

3. Result

3.1. Morphological Observation of Apoptosis under Inverted Microscope

After 24 hours, HeLa cells of untreated group were epithelial type; cell morphology presented flat or irregular or polygonal; the cell grew with adherence; the connection between the cells were more closely. However, HeLa cells of different concentrations of fullereneol and cisplatin showed different degrees of morphological changes, showing some cell bodies became smaller and smoother, burr-like membrane was shrunken; cells containing highly concentrated chromatin nuclei, chromatin condensation, and some have more increased cytoplasmic fragmented cells from adherent state off, suspended in a culture medium. HeLa cells of different concentrations of fullereneol combined with cisplatin showed that cell morphology were similar to the cisplatin group, while cell shrinkage were more apparent and the number of cells reduced obviously (Figures 1-4). With incubation time and the increase of drug concentration, the cells exfoliated increased gradually and the morphology changes more obviously. The fullereneol combined with cisplatin group, with synergies to strengthen the two drugs, the more obvious morphological changes that cell.

3.2. Growth Inhibition of Cisplatin on HeLa Cells

MTT analysis showed that the inhibition rate of cisplatin group (1 $\mu\text{g/mL}$) was higher than the control group



Figure 1. Control group ($\times 40$).



Figure 2. Cisplatin group ($\times 40$).



Figure 3. Fulleranol group ($\times 40$).

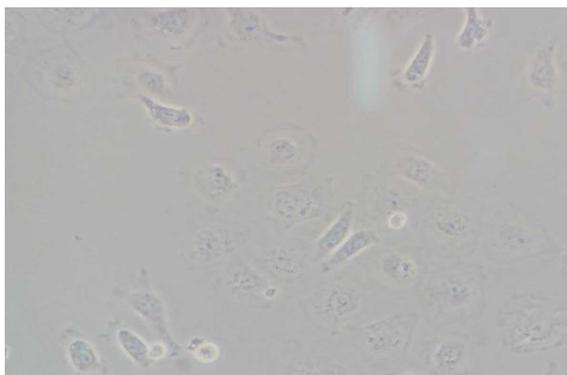


Figure 4. Combined group ($\times 40$).

from the beginning 24 h, and continued to 72 h. With the extension of time, the inhibition rate was significantly increased. After increasing the concentration of cisplatin, this trend is more obvious. Experimental results show that the growth of HeLa cells was significantly inhibited, the inhibition rate not only with the increase of drug concentration increased, but also with the extension of treatment time increased. Compared with the control group, treated with different concentrations of cisplatin in HeLa cells for 24 hours, 48 hours and 72 hours, the differences were statistically significant $P < 0.01$ (**Table 1**).

3.3. Growth Inhibition of Fulleranol on HeLa Cells

MTT analysis showed that the fulleranol group compared with control group, each concentration (2.5, 5.0, 10.0, $\mu\text{g/mL}$) of fulleranol was dealt with the 24 h, the inhibition rate began to rise and continued to 72 h; inhibition rate showed an increasing trend from low concentration (2.5 $\mu\text{g/mL}$) to the high mass concentration (10.0 $\mu\text{g/mL}$). After 24, 48, 72 h, cell inhibition rate was significantly lower than normal control group ($P < 0.05$). Thus informed that the growth of fulleranol on HeLa cells was more significantly, which inhibited the rate of inhibition with the drug concentration and prolonged increases. Compared with the control group, treated with different concentrations of fulleranol in HeLa cells for 24 hours, 48 hours and 72 hours, the differences were statistically significant $P < 0.01$ (**Table 2**).

3.4. Effect on HeLa Cells Growth Using Different Concentrations of Fulleranol in Combination with Cisplatin

Respectively, with three kinds of concentration of fulleranol (2.5, 5, 10 $\mu\text{g/mL}$) combined with cisplatin (5 $\mu\text{g/mL}$) on HeLa cell for 24, 48, 72 h, the result shown that fulleranol (2.5 $\mu\text{g/mL}$) combined with cisplatin, its inhibition rate have no difference compared with cisplatin alone, $P > 0.05$. When fulleranol (5, 10 $\mu\text{g/mL}$) combined with cisplatin (5 $\mu\text{g/mL}$), its inhibition rate was higher than the same application of cisplatin group and fullerenols group, the result was statistically significant, $P < 0.01$ (**Table 3**).

Table 1. Inhibition rate of different concentrations of cisplatin 24, 48, 72 hours on HeLa cells (Mean \pm SD, %).

Cisplatin dose	Treatment time		
	24 h	48 h	72 h
Control group	0.00 \pm 0.000	0.00 \pm 0.000	0.00 \pm 0.000
1.0 ug/mL	8.65 \pm 0.494 ^{**}	19.10 \pm 1.017 ^{**}	26.93 \pm 0.975 ^{**}
2.5 ug/mL	16.79 \pm 0.953 ^{**}	29.86 \pm 0.807 ^{**}	39.10 \pm 1.052 ^{**}
5.0 ug/mL	20.85 \pm 0.921 ^{**}	38.33 \pm 1.115 ^{**}	50.62 \pm 0.702 ^{**}
10.0 ug/mL	27.56 \pm 1.396 ^{**}	53.24 \pm 0.924 ^{**}	69.10 \pm 1.240 ^{**}

Note: ^{**}P < 0.01 compared with control group (0 μ g/mL).

Table 2. Inhibition rate of different concentrations of fullereneol 24, 48, 72 hours on HeLa cells (Mean \pm SD, %).

Fullereneol dose	Treatment time		
	24 h	48 h	72 h
Control group	0.0000 \pm 0.0000	0.0000 \pm 0.0000	0.0000 \pm 0.0000
2.5 ug/mL	0.0120 \pm 0.0014 [#]	0.0245 \pm 0.0021 [#]	0.0540 \pm 0.0042 [#]
5.0 ug/mL	0.2450 \pm 0.0042 [#]	0.2670 \pm 0.0028 [#]	0.2835 \pm 0.0021 [#]
10.0 ug/mL	0.2840 \pm 0.0014 [#]	0.3150 \pm 0.0042 [#]	0.3725 \pm 0.0078 [#]

Note: [#]P < 0.01 compared with control group (0 μ g/mL).

Table 3. Inhibition rate of different concentrations of fullereneol combined with cisplatin 24, 48, 72 h on HeLa cells (Mean \pm SD, %).

Fullereneol concentration with 5 ug/mL cisplatin in combination	Treatment time		
	24 h	48 h	72 h
Control group	20.850 \pm 0.9210	38.330 \pm 1.1150	50.620 \pm 0.7020
2.5 ug/mL	22.308 \pm 0.0065	40.113 \pm 0.1834	51.683 \pm 0.0082
5.0 ug/mL	22.326 \pm 0.0090 [▲]	40.150 \pm 0.0044 [▲]	51.707 \pm 0.0045 [▲]
10.0 ug/mL	22.349 \pm 0.0045 [▲]	40.171 \pm 0.0075 [▲]	51.724 \pm 0.0050 [▲]

Note: [▲]P < 0.05 compared with control group (cisplatin 5 μ g/mL).

4. Discussion

Apoptosis is a morphological changes, nuclear morphological changes which are typical characteristics of apoptosis, changes in cell morphology is thus reliably determine the standard apoptosis [10]. Chemotherapy based on cisplatin has become the standard chemotherapy in comprehensive treatment of cervical cancer. Some experiments have found that cisplatin can inhibit protein expression in HeLa cells of cervical cancer cell lines, promote cell apoptosis significantly and inhibit the growth to achieve a therapeutic effect, which is a certain age and dose effect relationship [11]. But drugs can cause serious adverse reactions, long-term large number of applications on the body can cause damage to normal tissue, and lead to cancer resistant to chemotherapy response. Fullerene C60 is a new ball-shaped molecule found in the 1980s, all composed of carbon atoms, it has a special hollow cage-like structure, the diameter of 0.71 nm. Fullereneol is a water-soluble derivative of fullerene C60, after the appropriate light intensity and irradiation time can fullereneol transitions from the ground state to an excited state, excited state fullereneol can be effective molecular oxygen quenching, resulting in free radical C60,

OH, O₂ or a single state oxygen ¹O₂, these free radicals and singlet oxygen O₂ can react with proteins, DNA and lipid film response, causing protein oxidation, DNA strand breaks, lipid peroxidation thereby destroying cancer structure of the cell membrane to induce apoptosis [12]. Professor Chen Dianbao and Professor Wang Zhijie of Wuhan University applied fullereneol -C60 (OH) n, C60 (OH) n/C70 (OH) n (n = 18 - 20) to carry on the growth inhibition test of human laryngeal cancer cell line Hep-2 and human cervical cancer cell line HeLa, observed the effects of apoptosis of cancer cells induced, and was first discovered in the dark light conditions, low concentration of fullereneol can also induce the apoptosis of cancer cells in a dose and time-dependent. The intensity of DNA staining in cancer cells treated with fullereneol decreased, the dead cells had the morphological characteristics of apoptotic cells and the cytoskeleton was severely damaged. [13]. Experiments have already made -C60 through the process of regulating autophagy to enhance the efficacy of chemotherapy and reduce the resistance of the biological function of cancer cells, which suggests a potential value of C60 becomes adjuvant chemotherapy [8]. Gordana Bogdanovi and other studies have shown that fullereneol can inhibit human breast cancer cells: T47D, MCF-7 cells and the activity of MDA-MB-231 in a concentration of 0.5 - 7.9 µg/mL, after 24, 48, 72 h cultivation, fullereneol maximum cytotoxicity of 40% - 45%, and fullereneol is a potent hydroxyl radical scavenger [14].

In this experiment, MTT assay showed weak proliferation inhibitory effect with fullereneol at a dose of 2.5 µg/mL on HeLa cells, when the dose was increased to when 10 µg/mL, the inhibitory effect on HeLa cells gradually increased and showed a time-dependent manner, that is to say, with increasing treatment time, the inhibition rate increased significantly, which was statistically significant. This experiment also shows cisplatin on the growth of HeLa cells was inhibited, and showed a dose-dependent and time-dependent manner. After this experiment, we choose moderate-dose cisplatin (5 µg/mL) to combine with fullereneol (2.5, 5, 10 µg/mL), after 24 h, 48 h, 72 h respectively, cell inhibition rates higher than fullereneol alone, so drug combination enhanced inhibition of human HeLa cells, there was no difference in addition of fullereneol with a concentration of 2.5 µg/mL. That compared with cisplatin alone, fullereneol with cisplatin significantly increases the effect of cisplatin, to achieve the same effect in reducing the use of lower amounts of cisplatin. Biological effects of fullereneol may be associated with ¹O₂ and OH- by system produce, then resulting in cell apoptosis, but the mechanism of action of the specific need further study.

5. Conclusion

In conclusion, systemic chemotherapy can improve the resection rate or radiosensitivity before surgery or radiotherapy in cervical cancer, but the side effects of acquired drug resistance of tumor cells often affect the overall effectiveness of chemotherapy, so finding a drug that can enhance the effectiveness of chemotherapy is the key to improve the success rate of chemotherapy and the survival rate of patients. Because of natural resistance and low toxicity characteristics of fullereneol, it has broad application prospects in cancer chemotherapy. The experimental results show that fullereneol has inhibitory effect and synergistic effect on cervical HeLa cells, which has a stronger anti-tumor effect, but due to its certain difference between the *in vitro* and *in vivo* drug interaction, it is necessary to further confirm the reliability, so that clinical treatment of cervical cancer tries new chemotherapeutic agents and regimens provide certain theoretical basis.

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