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Synoptic, apoptotic and cytotoxic effects of cisplatin and valproic acid compounds on ovarian cancer cells

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Abstract

Introduction: The anti-tumor effects of cisplatin and valproic acid have provided a new opportunity in the treatment of ovary tumor cell line.
Objectives: In this survey, the cytotoxic and apoptotic effects of cisplatin and valproic acid on the human ovary tumor cell line (ovar-3) was investigated.
Materials and Methods: In this study three groups were compared with the control group. Groups 1, 2 and 3 were studied with cisplatin, valproic acid and both of cisplatin and valproic acid respectively on ovary cell line (ovar-3). Each drug concentration was a spectrum between 1 to 1000 MM. The exposure time of ovar-3 ovary cells with the drugs was 36 hours. The MTT test for the live cell monitoring was performed with anexinPropidium iodide (PI).
Results: The IC50 for each cisplatin, valproic acid and the mixture of these compounds were 3.3 mm, 33 mm and 0.09 mm, respectively ($P<0.05$). The morphologic investigation for apoptosis showed that the mixture of cisplatin and valproic acid has the highest apoptotic effect to a level of 81% on ovar-3 cell line compared to each of these compounds.
Conclusion: Considering the significant synergistic apoptotic and lower cytotoxic effects of cisplatin and valproic acid drugs on ovary cells, the mixture of them can be an efficient anti-tumor compound targeting for treatment of ovar-3 cell line.

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Introduction

Ovary epithelial cancer is the most common type of ovarian cancer and usually remains asymptomatic during metastasis (1,2). For this reason, among all the malignancies of the female reproductive system, it has the highest mortality rate. The ovary cancer is the sixth female cancer in western countries and the fifth cause of death due to cancer among US women. Among all cancers in the female genitalia, ovarian malignancies have widespread clinical studies. Ovarian epithelial cancers, usually remain asymptomatic until metastasis. They are the most common ovarian malignancies in more than two-thirds of patients. When these tumors are diagnosed, they are in advanced stages of the disease. The cancer has the highest mortality rates among all female with genital herpes. There are more than 23 300 new cases of cancer in the United States each year, which 13900 women are expected to be victims of the disease. The risk of developing ovarian cancer during women's life is approximately 1.5%, and the risk of death due to ovarian cancer is approximately 1%. The factors that contribute to this cancer can be divided into two broad categories. A category such as infertility, obesity, excessive consumption of saturated animal fats and aging that increase the risk factors for developing this cancer. Another category, including multiple births, which reduces the risk of ovarian cancer by 50%, while pregnancy reduces the risk of developing this cancer by 13% to 19% and has a protective effect for this cancer (3). Cancer cells are recognized by uncontrolled growth and proliferation due to the uncontrolled function of the gene and proteins responsible for controlling the growth of the cell cycle (1). The most important causes of cancer

Key point

It is possible that synergistic apoptotic and lower cytotoxic effects of cisplatin and valproic acid drugs on ovary cells, and thus the mixture of them can be a more sufficient treatment target for treatment of ovar-3 cell line.

chemotherapy failure are relatively low efficacy, significant toxicity and drug resistance. Additionally, recurrence of cancer or the occurrence of metastatic disease in the lymph nodes (approximately 50% of cases) after a successful surgery are other causes of cancer chemotherapy failure (2,3). More than 70% of the patients when diagnosed are in the non-surgical stage. Hence, chemotherapy makes surgery more possible (4). Typically, single-drug regimens provide a response of about 20% and a lifespan of 4 months for patients, but over the past 2 decades, different chemo-therapeutic approaches have been used to treat advanced cancers. Despite several clinical trials in the field of advanced chemotherapy, an international standard diet is unavailable (5-7). Cisplatin is one of the most commonly administered drugs in cancer chemotherapy. This drug has been cured in the treatment of up to 85% of advanced cancer patients. The use of this drug in advanced ovarian cancer, bladder cancer, head and neck, stomach, lung cancer (non-small cell lung carcinoma) and in some types of neoplasms in children have led to a significant therapeutic response. The mechanism of the cisplatin cytotoxic effect is essentially linked to its binding to the nucleic acid nucleic acid (DNA) (8). In addition to cisplatin binding to DNA, cisplatin binding to other cellular components, such as proteins and some intracellular enzymes, has also been shown (9,10). Valproic acid is a short chain fatty acid which inhibits enzymes with histone deacetylases, thus increase acetylation of proteins, changes in chromosome structure and gene expression (11,12). This compound has been studied as an anti-cancer agent that can be administered either alone or in combination with other common cancer therapies, including chemotherapy and radiation therapy (13).

Objectives

Considering the prevalence of ovarian cancer and its high importance as one of the causes of mortality among women, the necessity of considering the treatment strategies of this disease is considered as one of the goals of this study. In the present study, the synergistic effect of cisplatin and valproic acid on the apoptosis of ovcar3 cell line was investigated.

Materials and Methods

Purchase of drugs

For this experimental study, cisplatin (CAS No. 99-66-1) and valproic acid (CAS No. 15663-27-1) were purchased from Sigma company.

Ovar-3 ovarian cancer cell lineage

The ovar3 cell line was obtained from the cell bank of the Iranian Center for Biological and Genetic Resources. Cells were cultured in DMEM medium containing 10% FBS in sterile conditions and incubated at 37°C, carbon dioxide concentration of 5% and relative humidity of 95%. Cells were prepared 3 times to prepare a cell line for the study of cytotoxicity.

Antibiotic preparation

Dissolve 1 g of streptomycin powder in 3.5 mL of water (H2O) to reach a final concentration of 285.7 µg/mL. The concentration of streptomycin in the culture medium should be 100 µg/mL. Therefore, to make 100 mL of culture medium, 35 µL streptomycin from the Stock was removed. Additionally, 1 g of penicillin powder was dissolved in 8 mL of water (H2O) to reach a final concentration of 500 000 U / ml. The concentration of penicillin in the culture medium should be 100 U/mL. Therefore, to make 100 mL of the culture medium, according to the calculations, 20 mL of penicillin should be taken from stock with a concentration of 500 000 U/mL.

Cell culture

The ovarian cancer cell line (Ovar-3) was developed from the cell bank of the Iranian Center for Biological and Genetic Resources. Ovar-3 cells were cultured in cell culture flasks (25 cm and 75 cm). Cells were cultured in RPMI-1640 enriched with FBS 10%, penicillin 100 units/mL, and streptomycin 100 µg/mL and kept in an incubator at 37°C and 5% CO_2. The medium was replaced every 2 days and the cells were re-cultured every 3 days. When the cells filled 80% of the flask (Confluent), the cells were tested.

MTT evaluation tests to detect the evolution of ovar-3 cells in the context of cisplatin and valproic acid

To calculate IC50, cisplatin and valproic acid alone, as well as a combination of two drugs were used. MTT test was used for 96 home plates. The 100 µL RPMI-1640 medium containing 10% FBS and 5000 cells per well was added to the incubator for 72 hours, incubated at 37°C and 5% CO2. After reaching the density of cells to 80% (growth logarithmic phase), the concentrations of cisplatin and valproic acid (1, 3.3, 10, 33, 100, 330 and 1000 µm) were added to each well (for each concentration of 3 wells), and 36 hours at 37°C were incubated in CO2, then 10 µL of MTT was added to each well, 5 mg/mL, and placed at 37°C for 5 hours. During MTN incubation, it is reduced by suction dehydrogenase and produces a crystalline form of formaldehyde. The color produced is directly related to the number of cells that are metabolically active. Formazan crystals must be soluble in DMSO prior to colorimetry. For this purpose, after completion of the incubation of the solution of each drain well, 200 µL DMSO plus 25 µL of Sorenson buffer was added. Then it was shaken slowly at room temperature for 10 minutes and the absorbance of each well was read by the ELISA reader at 570 nm. The absorption rate is proportional to the amount of live cells. Then the concentration-response curve was plotted and the IC50 of each of these compounds was calculated using Compusyn and Calcusyn software.

Apoptosis of ovar-3-grade cells in the presence of cisplatin and valproic acid

To determine the percentage of apoptotic cells in the treated

cell population and compare it with the cell population in negative control. The staining of cells with Annexin-V bundles was coupled to FITC and propidium iodide (PI). After treating the cells with different concentrations of drugs, the cells were trypsinized and next, were washed in phosphate buffered saline (PBS). To the cell centrifuge sediment, 100 µL of binding buffer was added to the 1.5 ml microtube. Following the addition of the buffering buffer, 10 µL of propidium iodide color and 5 µL of annexin dye were also added to the contents of the microtube. In the next step, the specimens were incubated at room temperature (25°C) for 10 minutes in the dark. Finally, for observing cell death or apoptosis using fluorescence microscopy and flow cytometry, 24, 48 and 72 hours were investigated.

Ethical issues

The research followed the tenets of the Declaration of Helsinki.

Statistical analysis

In the current study, CompuSyn and CalcuSyn software packages were used for statistical analysis.

Results

Determine IC50 of the cisplatin drug on the ovar-3 cell line

The percentage of ovarian oviduct (OD) was 0.9, 0.9 and 0.8, respectively, in the presence of the drug at a concentration of 1 mM in three replicates. The mean OD reading was 0.86 at three times, which indicates the cell's dynamics at a concentration of 1 mM. The level of the cell's toxicity at a concentration of 1 mM was determined by the formula below 0.54. It is worth noting that ovarian cell toxicity was determined at a concentration of 1 mM with OD of 0.9 for the control group.

The response curve for cisplatin and the logarithmic curve indicate that the concentration of the drug decreases the amount of toxicity and cell survival. To determine the IC50, cisplatin is a drug that causes 50% ovarian cellular inhibition. To determine the IC50 of the cisplatin drug, Excel software and CompuSyn were used to draw the curve. The IC50 cisplatin drug was obtained at various concentrations on the ovarian cell of 3.3 mM.

Determine IC50 of valproic acid on the ovar-3 cell line

The response curve for valproic acid and logarithmic curve showed that with increased drug concentrations, the level of toxicity and cell survival decreased. The IC50 value of valproic acid was 33 mM.

Combining cisplatin and valproic acid on the ovar-3 cell line

The ratio of valproic acid to the cisplatin drug was 10 to 1. Therefore, for valproic acid, concentrations of 1, 3.3, 10, 33, 100, 330 and 1000 mM were considered. The same concentration used in the single MTT test for the drug and for the cisplatin drug, the ratio 1 to 10 was combined with valproic acid, so the concentrations used for cisplatin were 0.1, 0.33, 1, 3.3, 10, 33 and 100 mM, respectively. The OD or the percentage of cellular dynamics derived from the combination of two drugs on ovarian cells is shown in Table 1. Although IC50 was not intended to be a combination of two drugs, the software was able to determine the IC50 of the two drugs, which was 0.09 mM. To obtain the CI (combination index), the combination of two drugs and the combination of the two drugs are incremental (=1), synergism (>1) or antagonism (<1), and to combine the two drugs to obtain the DRI (dose reduction index) combination of the two drugs is incremental (=1), synergism (>1) or antagonism (<1). CompuSyn software was used to get CI and DRI. The results of this study showed that CI was greater than 1 and DRI was less than 1. Therefore, the combination of two drugs on ovarian cancer cell lines had the property of synergism.

Evaluation of cell death of ovar-3 cells in the vicinity of cisplatin, valproic acid and combination of cisplatin and valproic acid

For the evaluation of apoptosis, medications were used alone and a combination of these concentrations was in the previous sections. For cisplatin, concentrations of 1 mM, 3.3 mM, 10 mM, 33 mM, 100 mM, 330 mM and 1000 mM and for valproic acid at concentrations of 1 mM, 3 mM, 10 mM, 33 mM, 100 mM, 330 mM and 1000 mM was used. The concentrations used for the combination of cisplatin and valproic acid were 100, 33, 103, 3, 1, 0.33 and 0.1 mM. The apoptosis of the cells was determined at 24, 48 and 72 hours. Apoptosis in control cells was 0.36%. In this study, induction of apoptosis by cisplatin and valproic acid alone and combined on ovar-3 cells was investigated by flow cytometry and staining with 10 µL propidium iodide color and 5 µL of annexin dye. The results showed that the concentration of 1 mM for cisplatin, 10 mM for valproic acid and the combination of cisplatin and valproic acid at 0.33 mM concentration had an apoptotic effect on ovarian cells. This measure was based on comparison with control group. The rate of ovarian cell tumor in

Table 1. The dynamics of ovarian cells obtained from ODs testing on the treated cells with combined valproic acid and cisplatin doses after 3 replications (n=3)

Dosage of combined cisplatin and valproic acid drug	Cell viability according to the OD
1	0.31
3.3	0.28
10	0.22
33	0.15
100	0.12
330	0.12
1000	0.51

each of the cases was individual, while in dose-dependent combination of drugs, the higher the dosage of the drug would be, the amount of cell death and thus cell decline will be observed. The apoptotic effect of the combination of two drugs was much higher than that of single cisplatin and valproic acid. In single mode, the amount of apoptotic property by cisplatin was much higher than valproic acid alone. The combination of two cisplatin and valproic acid drugs on ovar-3 cell apoptosis was compared to that of the control group. The results showed that the drug was fatal to 81%, which was 12% higher than cisplatin alone. Additionally, the severity of valproic Acid was 6.7% higher than the control group (0.36%)

The effects of growth inhibitory on ovarian cells after 24 hours are observed and time-dependent increases, hence they reach the maximum in 72 hours. Flow-cytometry analysis showed that control cells without any treatment showed 36% of the initial apoptosis and showed 0.09% of secondary apoptosis. In the case of ovarian cells treated with the combination of cisplatin and valproic acid, the percentage of apoptosis was 35.76% and the secondary apoptosis percentage was 45.24% (81% overall), while the amount of ovarian apoptosis was 69% using individual drugs, 69% for cisplatin and 6.7% for valproic acid. Morphological changes of ovarian cells were studied using light microscopy. Cells developed changes such as shrinkage, rounding and membrane irradiation after treatment with the combination of cisplatin and valproic acid. These morphological changes indicate induction of apoptosis.

Discussion

In this study, we used cytotoxic effects of two chemical drugs cisplatin and valproic acid alone and hybrid on the ovar-3 cell line. Some studies have shown that valproic acid produces a protective effect against apoptosis in some cancer cells, while its mechanism is not known exactly. Similar studies of this research have been carried out as well. Zendehdel and colleagues examined the changes in the chemical structure of the human A2780 ovarian cancer cell line biomolecules using Fourier transform infrared (FTIR), after an hour of exposure to various concentrations of cisplatin in a laboratory environment. A2780 cells were exposed at concentrations of 250, 100, 10, 0 and 500 µg/ml of cisplatin. The cellular toxicity of these cisplatin concentrations was measured by colony count. The FTIR spectrum of the cells was then evaluated using the Thermo Scientific™ OMNIC™ Series Software. Data were entered in Excel software and the results showed that cisplatin had a significant cytotoxicity (1 mg/mL) in IC50. In this study, the dose-response curve for cisplatin and valproic acid and the combination of two drugs indicates that, with increasing concentrations, cell survival decreases and the response curve increases logarithmically with increasing dosage. By drawing the CI and DRI curves and the logarithmic curve, the combination of the two drugs showed the properties of synergism. The results of this study demonstrated that CI was greater than 1 and DRI was less than 1. Therefore, the combination of two drugs on ovarian cancer cell lines had a synergistic effect. The results of this study were similar to those of the cisplatin drug in terms of efficacy. The IC50 concentration of the drug in this study was 3.3 mM. One of the reasons for this difference in the IC50 of the cisplatin drug was that the cell line in their study was the cell line of ovarian A2780 cells, while in the present ovary cell line was ovar-3 ($P<0.05$). So far, no similar study has been conducted to evaluate the effect of valproic acid on ovarian cell lines in the country. A similar study examined the effect of valproic acid and radiation therapy on MCF-7 cell line viability. In this descriptive-analytical study, MCF-7 (breast cancer cells) cells were treated with different concentrations of valproic acid alone and in combination with different doses of radiotherapy.

After screening for cytotoxicity with a neutralized staining test, the closest results were selected for LD50. The cells were then subjected to 3 concentrations of valproic acid (2, 8 and 16 mM) and Gy 4 doses of radiation therapy, and the vitality of the cells was examined by Trypan blue staining. The closest concentrations of LD50 from doses of 1, 2, 4, 8, 16, 32, 64 and 128 mM valproic acid were administered in combination with various doses of 0.5, 2, 4, 6 and 8 mg radiotherapy at 2, 8 and 16 mM valproic acid and dose of Gy 4 (radiation dose). In addition, cell viability was dependent on valproic acid concentration ($P<0.05$). We found that valproic acid, both alone and in combination with radiation therapy, significantly reduced cell viability. However, in combination, it has an increasing effect on cell viability (14) which was similar to results of our study. Valproic acid had significant potential for ovar-3 cell survival ($P<0.05$). This study showed that co-administration of this drug against ovar-3 cell line compared with the use of any of the drugs. The single-stranded effects were more pronounced and the IC50 concentration of these two drugs was found to be 0.09 mM. Statistically, the association between the combination of cisplatin and valproic acid with ovar-3 cell viability was significantly higher than that of each drug alone ($P<0.001$). According to the results of CI and DRI, the results showed that the combination of two drugs - cisplatin and valproic acid - had an inhibitory synergistic effect on ovarian cells. To determine the apoptosis of ovar-3 cells in the presence of chemical drugs, concentrations lower than IC50 were determined as apoptosis indices. The combination of cisplatin and valproic acid showed a significant synergistic effect on apoptosis of ovar-3 cells ($P<0.001$). In this study, flow cytometry analysis showed that control cells without any treatment exhibited 36% of initial apoptosis and 0.09% secondary apoptosis. In the case of ovarian cells treated with the combination of cisplatin and valproic acid, the percentage of apoptosis was 35.76% and the secondary apoptosis percentage was 45.24% (81% overall). However, the amount of ovarian apoptosis was 69% for single drugs, 69% for cisplatin and 6.7% for valproic acid. The results of

this study were consistent with the study by Najafzadeh et al, in which effect of retinoic acid and its combination with cisplatin was assessed on gastric cancer cell survival (AGS) (15). Cisplatin is one of the most effective treatments for treating many cancers that cause apoptosis by cross-linked DNA. In this study, the gastric cancer cell line was cultured in RPMI-1640 medium and then the effect of different dilutions of retinoic acid and cisplatin on these cells was evaluated by colony and colorimetric methods of acridine and ethidium bromide. The results showed that retinoic acid whole transfusion alone did not have a significant effect on the death of the gastric cancer cell line, but the combination of retinoic acid and cisplatin increased cell necrosis and apoptosis. Apoptosis was observed in cells treated with 10 µM retinoic acid, all-trans and 5 and 10 µg cisplatin compared to those treated with a drug ($P<0.001$) (15). In this study, the effect of treatment with antimiR-182 on the increase of sensitivity to cisplatin in HeLa cells was investigated. After culture of HeLa cells by adding different doses of cisplatin and determining the mortality rate of cells by MTT, the LD50 value of the drug was obtained. Then, the optimal transfection and optimum concentration of anti-miR-182were calculated. Finally, anti-miR-182 and cisplatin were transferred separately and simultaneously to HeLa cells, and the rate of reduction in cell proliferation and the increase in apoptosis induced by inhibition by anti-miR-182 alone and along with cisplatin by MTT and flow cytometry were evaluated.

Conclusion

In general, the results of this study have similarities with previous studies in the spectrum of synergistic effects, but those reported IC50 levels and concentrations of compounds inducing apoptosis are very different. One of the important reasons for the difference could be the type of cancer cells which was studied. Other reasons may be the methodology, the chemical compounds, the length of time the cell was treated with the drug, the number of cells adjacent to each of the compounds studied, the type of cancer cells and at what stage, and the range of drug concentration individually or in combination. In general, the results of this study showed that each of the cisplatin and valproic acid drugs and the combination of them were effective on the ovar-3 ovarian cells and this relationship was statistically significant ($P<0.05$). The combination of two drugs has generally been effective in reducing cell biopsy and inducing apoptosis in ovar-3 cells. Cisplatin appears to be highly effective in inducing valproic acid activity against ovarian cells. DRI and CI values from combination of two drugs confer synergism regarding ovarian cells.

Authors' contribution

FF and VN conducted the research and contributed to the manuscript equally. All authors read and signed the final paper.

Conflicts of interest

The authors declare that there are no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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