THE EFFECT OF IONIZING RADIATION IN COMBINATION WITH CHEMOTHERAPY DRUG DOXORUBICIN ON A431 HUMAN EPIDERMOID CARCINOMA CELL TREATMENT

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Abstract. Radiotherapy is one of the most effective and most commonly used methods of cancer treatment. However, as a result of irradiation, there are side effects that occur as a result of ionizing radiation on healthy tissues. The use of a combined approach with the use of low doses of radiation and antitumor drugs that have a radiosensitizing effect may be one of the ways to reduce side effects and overcome the resistance of malignant cells. This study was undertaken to evaluate the combined effects of radiotherapy and the chemotherapeutic agent Doxorubicin on A431 human epidermoid carcinoma cell line. The cells were incubated with the antitumor antibiotic Doxorubicin and then exposed to high-energy electron ionizing radiation. The cell viability was examined using the MTT assay. The results showed that Doxorubicin acts as a radiosensitizer. Moreover, the combined effect of Doxorubicin and high-energy ionizing radiation of electrons is additive. According to the obtained results, combination therapy used in the treatment of oncological diseases can significantly reduce the radiation dose and minimize the side effects that occur during high doses of irradiation.

Keywords: ionizing radiation, Doxorubicin, combination therapy, additive effect.

List of Abbreviations

CI – Combination index

DMEM – Dulbecco's Modified Eagle Medium

DNA – Deoxyribonucleic acid

DOX – Doxorubicin

Introduction

Today, more than half of cancer patients are treated with ionizing radiation as an adjuvant, neoadjuvant, or palliative treatment at some point in the clinical course of the disease (Baskar *et al*., 2012; Rubin *et al*., 2005). Radiation therapy is used in the treatment of various malignant neoplasms, including basal cell cancer and squamous cell cancer, head and neck, breast, cervical, prostate and other cancers (Mancuso *et al*., 2012; Samarasinghe & Madan, 2005). Despite the fact that ionizing radiation targets the tumor, healthy cells are also inevitably exposed to radiation, including non-irradiated neighboring cells (bystander effect) and even more distant cells (abscopal effect) located outside the primary tumor irradiation field (Daguenet *et al*., 2020; Mancuso *et al*., 2012). The side effects caused by radiotherapy on healthy cells can be minimized if the radiation dose is reduced while maintaining the overall therapeutic efficacy. It is known that the use of only one method of antitumor treatment is often insufficient to contain a malignant tumor, since malignant cells use different ways to start the process of carcinogenesis (Park *et al*., 2020). While radiation therapy provides local control of the primary tumor, the added systemic treatment has the potential to treat latent distant disease and provide additional radiosensitization benefits. In this regard, combined treatment using radiation and systemic therapy is currently the cornerstone of anticancer treatment. However, despite the widespread use of various combinations, researchers are at the stage of finding the optimal combination of agents to minimize side effects and maximize therapeutic benefit.

Radiosensitizers are chemical or pharmaceutical agents that can enhance the killing effect on tumor cells by accelerating DNA damage and indirectly generating free radicals (Negi *et al*., 2016). Clinically approved cytotoxic chemotherapy drugs such as cisplatin, vinorelbine, doxorubicin and others used as radiosensitizers

are of particular interest in recent years (Aghaee *et al*., 2013; Chen *et al*., 2021).

Doxorubicin (DOX) is a member of the cytotoxic anthracycline antibiotics, a group of antibiotics that appear to have antimitotic and antiproliferative effects (Tacar *et al*., 2013). Some studies have convincingly shown that low doses of Doxorubicin with the least cellular toxicity can be an effective treatment when used in combination with ionizing radiation (Aghaee *et al*., 2013; Lee *et al*., 2005; Popescu *et al*., 2021; Yazbeck *et al*., 2022). For instance, the combined effect of Doxorubicin and low doses of γ-radiation from Co-60 and Cs-137 sources, as well as X-rays on cervical cancer HeLa cells and various human breast cancer cell lines: SKBR3, MCF-7 and T47D (Aghaee *et al*., 2013; Jagetia & Nayak, 2000; Zamulaeva *et al*., 2015). It was shown in the study (Kandil & Aziz, 2016) that administration of Doxorubicin and subsequent fractionated irradiation of tumor-bearing mice with γ-radiation from a Cs137 source resulted in a significant inhibition of tumor growth as early as two weeks after the start of therapy.

In this manuscript, we report on *in vitro* studies of the combined effects of high-energy electron ionizing radiation and the chemotherapy drug Doxorubicin on A431 human epidermoid carcinoma cells.

Material and Methods

Cells

We used a cell culture of human epidermoid carcinoma A431, which is widely treated with radiotherapy in clinical practice (Bichakjian *et al*., 2016; Bonerandi *et al*., 2011). The cells were cultured in 25 cm^2 culture flasks (Corning, USA) at 37 °C and 5% CO_2 in air. DMEM (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone, USA) and 2 mM L-glutamine (PanEco, Russia) was used as a nutrient medium. The cells were removed from the culture flask using a trypsin-EDTA solution (1:1) (PanEco, Russia), and 10 mM phosphate-buffered saline (PBS) was used to wash the cells. The cells were transplanted when the culture reached 80% confluence.

Drug / Medication / Agent / Preparation

As a cytotoxic agent, we used the approved chemotherapy drug Doxorubicin (Pharmachemy B.V., the Netherlands), a cytotoxic anthracycline antibiotic isolated from a culture of Streptomyces peucetius var. caesius (Rivankar, 2014).

Sources of ionizing radiation

Irradiation was performed using a Novalis Tx linear accelerator (Varian, USA) with an electron energy of 6 MeV. SSD was 100 cm, the field size was 25x25 cm^2 and dose rate was 10 Gy/min. The radiation dose was controlled by the exposure time, which did not exceed 8 minutes. The calculation of the delivered dose was performed using the Electron Monte Carlo eMC algorithm.

Evaluation of cytotoxicity in monotherapy

In order to select doses of ionizing radiation and DOX concentrations for subsequent combined use, cells were planted in 96-well culture plates (Corning, USA) at a concentration of 5,000 cells per well. After 12 hours, after the cells were attached to the substrate, they were treated with irradiation and DOX.

Irradiation with ionizing radiation was carried out in doses of 4–16 Gy, the radiation dose was controlled by time.

DOX was added to cells in serum-free culture growth medium at concentrations of 0.01–100 mM and incubated for 1 hour. After the end of the incubation, the medium was replaced with a complete growth medium.

Viability was assessed in all treatment options 24 hours after exposure by the microtiter test (the MTT assay) (Kumar et al., 2018). For this, 3(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT reagent, Alfa Aesar, UK) was added to the growth medium to a final concentration of 0.5 mg/ml and incubated for 4 hours at 37°C and 5% CO_2 in the air. Next, the incubation medium was taken, and the crystals of the formed colored MTT-formazan were dissolved in 200 μl of dimethyl sulfoxide. The optical density of the contents of each well was measured on an EMax Plus Microplate spectrophotometer (Thermo Fisher Scientific,

USA) at a wavelength of 570 nm. Cell viability was assessed by the ratio of the optical density of the formazan solution in each sample to the control sample.

Evaluation of cytotoxicity in combination therapy

To assess cytotoxicity in combination therapy, similarly to monotherapy, cells were planted in 96-well culture plates (Corning, USA) at a concentration of 5,000 cells per well. Twelve hours after cells were attached to the substrate, DOX was added to the cells in serum-free growth medium at concentrations of 5 or 10 mM and incubated for 1 hour. After the end of the incubation, the medium was replaced with a complete growth medium. Irradiation with ionizing radiation was performed immediately after the end of incubation at doses of 4 and 16 Gy. Viability was assessed 24 hours after exposure by the microtiter test (the MTT assay) (Kumar *et al*., 2018). The scheme of the experiment is shown in Figure 1.

The combination index (CI) was used to determine the intensity of the combined effect (Ianevski *et al*., 2017). The CI was calculated according to the following formula:

$$((E_a + E_b) - E_a \times E_b)/E_{ab}, \text{ where}$$

E_a is the proportion of dead cells as a result of ionizing radiation;

E_b is the proportion of dead cells as a result of the cytotoxic drug treatment;

E_{ab} is the proportion of dead cells as a result of combination therapy.

The index values within the following frame $0.9 < CI < 1.1$ indicate an additive effect, values below 0.9 indicate synergy.

Statistical analysis

Statistical analysis was carried out using GraphPad Prizm v.9.0 software (GraphPad, USA). Two-way ANOVA (the Tukey test) was used as a criterion for significance.

Results

Cytotoxicity of DOX and ionizing radiation of high energy electrons in relation to A431 cells in monotherapy

To assess the combined effect of DOX and ionizing radiation, we chose an irradiation regimen that is clinically used in external beam radiation therapy.

The study has shown that the viability of A431 human epidermoid carcinoma cells after exposure to high-energy electron ionizing radiation at doses of 4 to 16 Gy was reduced by 20% (Fig. 2A). The studied dose range is not sufficient for monotherapy, since it does not have considerable therapeutic effects, however, it can be used for combination therapy, since it can potentially reduce the risk of side effects from radiation therapy (Dawood *et al*., 2021).

DOX cytotoxicity was assessed in the concentration range of 0.01–300 mM. The concentration of DOX inhibiting cell growth by 50% (the IC50) was 10 mM (Fig. 2B). Since it is known that the use of high concentrations of DOX is associated with chronic side effects, to assess the combined effects, we chose concentrations not exceeding the IC50, namely 5 and 10 mM.

Cytotoxicity of DOX and ionizing radiation of high energy electrons in relation to A431 cells in combination therapy

The study has shown that when incubating A431 human epidermoid carcinoma cells with DOX and subsequently irradiating them with high-energy electron ionizing radiation, there is a significant decrease in viability compared to both variants of monomodal exposure (Fig. 3).

Incubation of cells with 5 mM DOX followed by irradiation at a dose of 4 Gy led to a decrease in cell viability by 40%, the CI was 0.9, which indicates an additive effect (Table 1). A similar degree of combined effect is observed with a combination of 5 mM DOX and a dose of 16 Gy. Cell viability for this combination was reduced by 50%, and the CI reached 0.9.

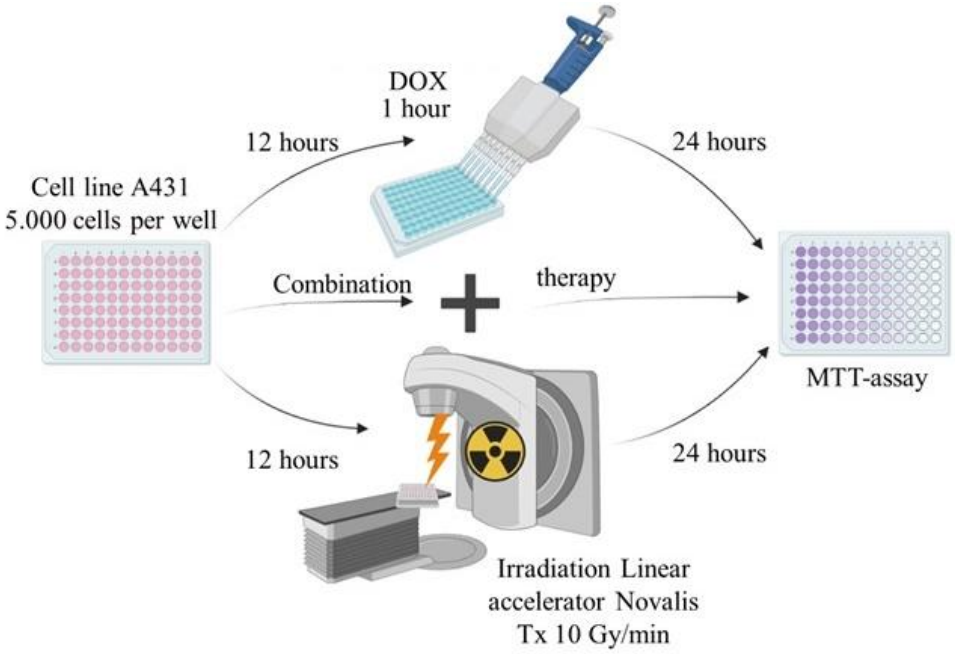

Fig. 1. Experiment scheme

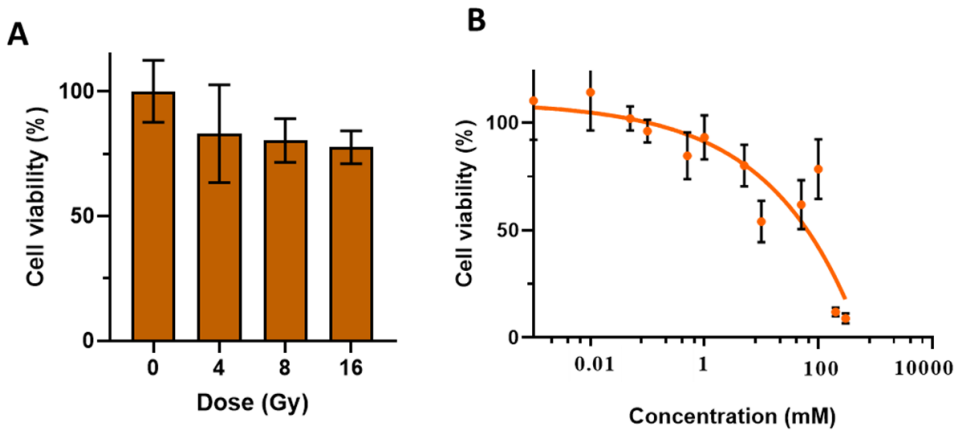

Fig. 2. Viability of A431 cells 24 hours after monotherapy. A – Exposure to high-energy ionizing radiation; B – Incubation with DOX. Error bars represent the standard deviation (SD)

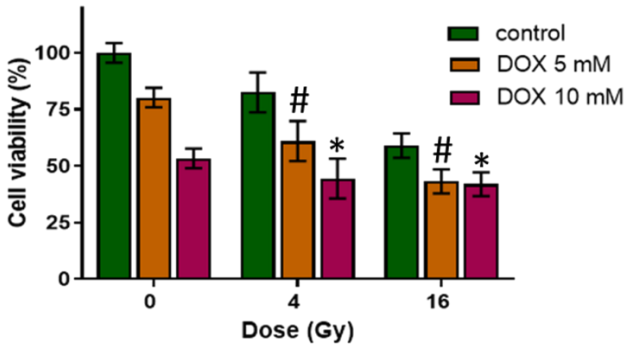

Fig. 3. Viability of A431 cells 24 hours after irradiation with a source of ionizing radiation in high dose rate mode, incubation for 1 hour with Doxorubicin and their combined action. Error bars represent the standard deviation (SD). * P-value < 0.5; # P-value < 0.01

Table 1

Combination index

Dose, Gy	DOX 5 mM	DOX 10 mM
4 Gy	0.9	0.9
16 Gy	1	1.2

Note: * green – additive effect; red – no combined effect

Incubation of cells with 10 mM DOX and subsequent irradiation at a dose of 4 Gy also led to a decrease in cell viability by 50%, but the CI was 1, which indicates a less pronounced additive effect than in previous treatment options. With incubation with 10 mM Doxorubicin and subsequent irradiation at a dose of 16 Gy, no combinative effect was registered, the CI was 1.2.

Discussion

Methods and approaches to cancer radiotherapy have evolved over decades, which ultimately contributed to a decrease in the severity of treatment and an increase in the cancer patients' quality of life and its duration (Hosseinzadeh *et al*., 2017). Despite the fact that from a technological point of view, the method of radiation therapy has made significant progress, the negative side effects caused by ionizing radiation in relation to normal dividing cells cannot be ignored. In addition, clinical experience strongly suggests that a single treatment regimen is not sufficient to contain malignant growth (Olivares-Urbano *et al*., 2020). Combining several antitumor strategies can increase the effectiveness of radiotherapy compared to a monotherapy approach, as it acts on key pathways of carcinogenesis in a synergistic or additive manner (Lu *et al*., 2021).

Our study has shown that when exposed to ionizing radiation at doses of 4–16 Gy, cell viability decreases by 20%. It is known that the damaging effect of ionizing radiation on cells is based on the formation of reactive oxygen species that occur during water radiolysis, which cause DNA damage, including base modification, changes in deoxyribose, and single- and double-strand breaks. In addition to indirect DNA damage by reactive oxygen species, ionizing radiation also causes direct DNA damage (Cheok *et al*., 2021). The chemotherapeutic drug Doxorubicin was chosen as an agent for radiosensitization of tumor cells. For the studied cell line, the IC50 of Doxorubicin reached 10 mM. A number of studies have shown the effectiveness of the combined use of DOX and ionizing radiation. The main pool of research work in this area is made using sources of electromagnetic ionizing radiation: γ-radiation sources such as Co-60 and Cs-137, and X-rays. Human breast cancer cells have been studied as tumor models. The conducted studies convincingly showed the additive and subadditive effects in the selected ranges of doses and concentrations.

The chemotherapeutic drug Doxorubicin was chosen as an agent for radiosensitization of tumor cells. For the studied cell line, the IC50 of Doxorubicin reached 10 mM. A number of studies have shown the effectiveness of the combined use of DOX and ionizing radiation. The main pool of research work in this area is made using sources of electromagnetic ionizing radiation: γ-radiation sources such as Co-60 and Cs-137, and X-rays. Human breast cancer cells have been studied as tumor models. The conducted studies convincingly showed the additive and subadditive effects in the selected ranges of doses and concentrations. (Tewey *et al*., 1984). The main mechanism of the damaging effect of ionizing radiation consists in single- and double-strand DNA breaks during direct and indirect action (Baskar *et al*., 2012; Zander *et al*., 2019). Potentially, the additive effect will allow to reduce the radiation dose and the total radiation load on the body while maintaining the overall therapeutic efficacy.

Despite the fact that the exact mechanism of combination therapy with Dox and ionizing radiation is not currently known, in our opinion, these findings deserve further study and can form the basis for the development of protocols for combined antitumor treatment.

Conclusion

The combined use of the antitumor antibiotic Doxorubicin in combination with ionizing radiation effectively reduces the viability of A431 human epidermoid carcinoma cells. In our study, we observed an additive effect of the combination of Doxorubicin and exposure to high-energy electron ionizing radiation. Compared with monotherapy, such combination therapy, may have the potential advantage ensuring less damage to normal cells and a reduction in overall toxic effects on the body. The exact mechanism of this combination therapy is not clear and may be worth elucidating in the future. These effects deserve further study in order to determine their role in combined radiation therapy.

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