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Sensitization of rat C6 glioma cells to ionizing radiation by porphyrins

Laimutė Rutkovskienė[1],

Laima Plėšnienė[1, 2],

Daiva Sendiulienė[1],

Alfonsas Stančius[1],

Jurgita Liutkevičiūtė-Navickienė[1]

[1] *Institute of Oncology, Vilnius University, Vilnius, Lithuania*

[2] *Klaipėda University, Klaipėda, Lithuania*

Background. Radiosensitizers are used in order to increase the efficacy of radiotherapy. Most of the presently known radiosensitizing agents have a poor selectivity and are not tumour-specific. Porphyrins have a selective uptake in tumour relative to the surrounding normal tissue. The aim of the present work was to test the capability of two photosensitizers – hematoporphyrin derivative (HpD) or temoporfin (mTHPC) – and gamma rays to produce some kind of selective inhibition of tumour cell proliferation.

Materials and methods. Dark toxicity experiments were carried out using a sensitizer concentration range 0–50 μg/ml for HpD, or 0–5 μg/ml for mTHPC. For the radiosensitized treatment of rat C6 glioma cells, HpD was added at a final concentration of 1 μg/ml and mTHPC at a final concentration of 0.1 μg/ml. The irradiation with gamma rays was performed using doses ranging from 0 to 8 Gy. Cell survival was determined using the colony forming assay.

Results. HpD (1 μg/ml) and mTHPC (0.1 μg/ml) were found to have no toxic effects on C6 glioma cells. A cytotoxic dose without drugs, inducing a reduction in colony survival by 20%, was achieved at 2 Gy and by 50% at 4 Gy. The radiosensitized treatment of cells with HpD resulted in a significant ($p \leq 0.05$) decline in cell survival as compared with irradiation alone. For C6 treated with mTHPC, the results did not differ between the two groups (with and without the drug).

Conclusions. The results of this study have shown that mTHPC (0.1 μg/ml) does not act as a radiosensitizer, whereas HpD can act, under certain conditions, as a tumour radiosensitizer. These findings suggest that HpD is a potential agent in combination with radiation therapy of malignant gliomas.

Key words: rat C6 glioma cells, radiosensitization, porphyrins, colony forming assay

INTRODUCTION

Primary brain tumours are among the ten most common causes of cancer-related death (1). Malignant gliomas, the most common type of brain tumours in adults, present a remarkable degree of neovascularisation and invasiveness into the surrounding tissues. These features account for their poor prognosis, and although combinations of surgery, radiotherapy and chemotherapy with temozolomide are used, more than 90% of the patients experience local recurrence and their survival is extremely low (2–4). Unfortunately, the therapeutic efficacy of radiation in these tumours is limited by either intrinsic or acquired cell resistance (5). These facts stress the need for more effective therapeutic strategies (6). Extending the traditional treatment armamentarium, photodynamic therapy (PDT) has been under investigation as a possible adjuvant for glioma therapy. This mode of treatment relies on the selective uptake or retention of intravenously administered photosensitizing dyes, such as hematoporphyrin derivative (HpD), in malignant brain tissue. PDT utilises photosensitizing compounds which apparently accumulate in malignant tumour

Correspondence to: Laimutė Rutkovskienė, Institute of Oncology, Vilnius University, Santariškių 1, LT-08406 Vilnius, Lithuania. E-mail: laimute.rutkovskiene@vuoi.lt

tissue. When exposed to light of an appropriate wavelength and energy, tumours are selectively damaged (7, 8). The blood-brain barrier excludes large drug molecules, such as photosensitizers, from the brain. They will be found, however, in tumour tissue where there is no such barrier. In this way, exposure of the normal brain tissue to photosensitizers is low, while at the same time the photosensitizer concentrates in the tumour (9). The tumour-to-brain ratio of HpD has been reported to be as high as 50 : 1 (10, 11).

On the other hand, PDT is unsuitable for the treatment of deep-seated tumours because the penetration of such tissues by light is poor, even when light from the red region of the spectrum is employed. As an approach to overcome this disadvantage, many studies have been carried out to determine whether different photosensitizers sensitize cells and tumours to ionizing radiation. The results of these studies, however, have been contradictory (12–16). Since Figge (17) first reported hematoporphyrin to be able to efficiently radiosensitize paramecia to ionizing radiation, it has been shown that porphyrins sensitize mammalian cells and tumours to ionizing radiation (18, 19). Other investigators did not observe radiosensitization effects by porphyrins in their *in vitro* studies. In contrast, Zhang et al. (5) reported a significant sensitization due to hypericin on human malignant glioma cells exposed to ionizing radiation (2–6 Gy). Kostron et al. (19) found an interaction of HpD with light and ionizing radiation in a rat glioma tumour model.

The choice of C6 cells was dictated by the following considerations: 1) this cell line is a classic model of glial-derived tumours which present an enormous therapeutic challenge since most of them cannot be cured by surgery, radiotherapy or chemotherapy (20); 2) to our knowledge, this is the first report of HpD-mediated radiosensitization of C6 glioma cells *in vitro*.

Morphologically, C6 glioma is similar to glioblastoma multiforme (GBM) when injected into the brain of neonatal rats. C6 glioma cell line is a useful experimental model system for the study of glioblastoma growth, invasion and metastases. In addition, these cells allow the screening for future drug targets and for the development of novel therapies (21). Histologically, the C6 cell line has been classified as an astrocytoma, and this cell line demonstrated the greatest number of genes whose expression was similar to that reported for human brain tumours (22).

In the present study, we decided to investigate the capability of a hematoporphyrin derivative to act on growth inhibition and radiosensitization of cultured glioma cells from the therapeutic standpoint. We show here that the hematoporphyrin derivative sensitizes C6 glioma cells to ionizing radiation, suggesting that HpD may be a useful drug in combination therapy for patients with malignant gliomas (23).

MATERIALS AND METHODS

Materials. The hematoporphyrin derivative (5 mg/ml in 0.9% saline) was obtained from Photogem Company (Moscow, Russia), and was kept at 4 °C in the dark until use. *Meso*-tetrakis(3-hydroxyphenyl)-chlorin (mTHPC, temoporfin) was kindly provided by R. Bonnett (London University, UK). mTHPC was dissolved in ethanol as a 1 mg/ml stock solution and stored at –20 °C in the dark. All experiments were performed using dilutions of the stock solutions with cell incubation media. All other reagents were from Sigma unless stated otherwise.

Cell culture. Culture flasks and Petri dishes were from Techno Plastic Products AG. Fetal calf serum (FCS) was from Gibco BRL. Other tissue culture products were purchased from Sigma.

Rat C6 glioma cells were obtained from the Institute of Cytology, Sankt-Petersburg, Russia. The cells were cultured in Dulbecco's minimal essential medium (DMEM) with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin, and 2 mM glutamine. The cells were routinely grown in monolayer in 25 cm^2 tissue culture flasks and incubated at 37 °C in a 5% CO_2 environment. C6 cells were subcultured by dispersal with 0.025% trypsin in 0.02% ethylene diamine tetra-acetic acid (EDTA) and replated at a 1 : 10 dilution three times per week to maintain cells in the exponential growth phase. The average doubling time for C6 cells was 26 hours (6).

Dark toxicity. Cell exposure to sensitizers was carried out in 9.2 cm^2 Petri dishes. The cells were seeded out as a suspension in DMEM supplemented with 10% FCS at a density of 0.2×10^6 cells/ml in 1.85 ml per dish. Eighteen hours later, after changing the medium, different sensitizers were added for a specified duration. To determine the influence of HpD and mTHPC on C6 glioma cells in the absence of light, the attached cells were washed three times with PBS. HpD was added to yield a range of final concentrations, typically spanning within 0–50 μg/ml, and mTHPC was added (0–5 μg/ml). When handling the samples containing sensitizers, precautions were taken to avoid irradiating the samples with room light by reducing the sources of illumination to a minimum and by protecting the samples from light with aluminium foil. The cells were incubated at 37 °C in a 5% CO_2 environment respectively for 24 h and 18 h. Cell number was determined using crystal violet (CV) dye. The medium was removed, cells were fixated with 96% ethanol for 10 min and stained with 50 μl of 0.5% crystal violet in 20% ethanol for 30 min at room temperature. The plate was then washed 10 times with tap water and dried at room temperature. The dye was eluted with 2 ml 0.1% acetic acid in 50% ethanol. The absorbance at 585 nm was determined using a spectrophotometer (Perkin Elmer $\lambda = 20$, USA).

Cell treatment. An initial inoculum of 0.2×10^6/ml cells was used for each culture flask containing 5 ml

of growth medium. Eighteen hours later, after changing the medium, different sensitizers were added for a specified duration. For radiosensitized treatment of cells, HpD was added to the serum-free DMEM at a final concentration of 1 μg/ml and mTHPC – at a final concentration of 0.1 μg/ml. These concentrations were chosen after toxicity tests with the different concentrations of sensitizers. The cells were cultured into 25 cm^2 flasks wrapped in aluminium foil and incubated in the dark at 37 °C in a 5% CO_2 environment respectively for 24 h and 18 h. The medium with an extracellular sensitizer was then removed by rinsing the cell monolayer 2 times with room temperature PBS and replaced with fresh medium free of drug in near darkness. The cells were irradiated in monolayers with gamma rays using ^{60}Co source (AGAT-R1, Kharkiv, Ukraine) at a dose rate of approximately 1.1 Gy/min. The irradiation was performed using doses ranging from 0 to 8 Gy, administered in a single fraction. Controls receiving either sensitizer alone, radiotherapy alone, or neither were obtained for C6 cells. Generally, each experimental condition was replicated three times.

Clonogenic survival assay. After experimental treatments, C6 cells were processed for the clonogenic assay. Therefore, the monolayers were briefly treated with trypsin-EDTA, and the cells were centrifuged at 1000× g for 10 minutes. After aspiration of the supernatant, the cells were resuspended in phosphate-buffered saline (PBS) and counted with a cell counter and an analyser system (Innovatis AG Casy® Technology, Reutlingen, Germany). From the suspension, 600 cells were seeded onto 100 mm tissue culture in dishes and incubated at 37 °C for 9–10 days. Control dishes were treated with different doses of radiation, and the plating efficiency was determined by treating cells with the medium alone. The plating efficiency was calculated as follows:

$$\text{Plating efficiency} = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times 100.$$

The colonies were stained with crystal violet. Colonies with diameters of 2 mm and larger (>50 cells) were subsequently counted with the aid of a light box, using an electronic counting pencil (Colony Counter; Bio, Kobe, Japan). Experiments were repeated at least three times to obtain the mean and SE. The radiation survival fraction was then calculated as

$$\text{Survival fraction} = \frac{\text{Number of colonies in radiated cells}}{\text{Number of colonies in control}} \times 100.$$

Statistical analysis. The significance of variability between the results from each group and the corresponding controls was determined by unpaired t-test or ANOVA. All results are expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. The P value less than 0.05 was considered significant.

RESULTS

Dark toxicity. The dark toxicity experiments were carried out using a sensitizer concentration range of 0–50 μg/ml for HpD, or 0–5 μg/ml for mTHPC. The dark toxicity of HpD on C6 glioma cells is shown in Fig. 1.

No significant C6 cytotoxity was detected at the HpD dose of 1 μg/ml. However, with HpD doses of 2.5 μg/ml and 5 μg/ml, a significant cytotoxic response was present: 71.2 ± 5.9% ($p < 0.05$) and 49.1 ± 7.3% ($p < 0.05$) of control, respectively.

The dark toxicity of mTHPC on C6 glioma cells is shown in Fig. 2.

HpD is less toxic to glioma cells than mTHPC in the absence of light, with a 50% cell survival (CS_{50}) = 5 μg/ml as compared with the corresponding value for mTHPC of 1 μg/ml. respectively. No significant C6 cytotoxity was detected at the mTHPC dose of 0.1 μg/ml.

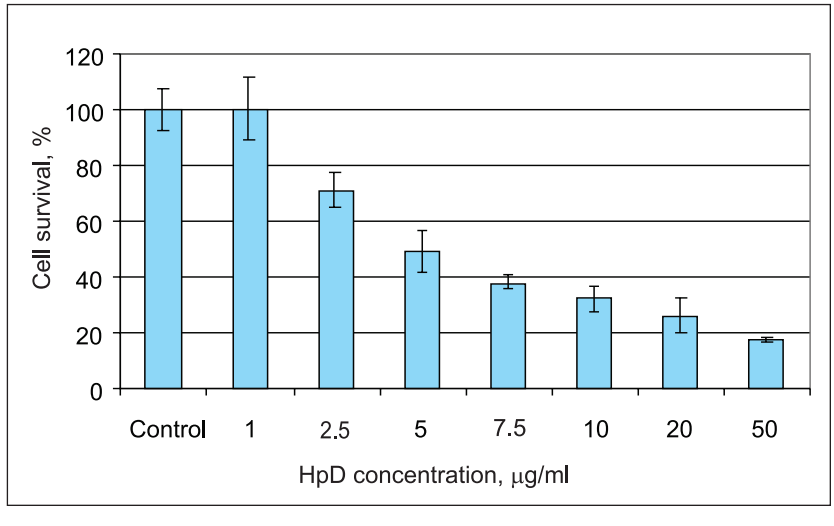

Fig. 1. Dark toxicity of HpD to C6 cells as determined by CV assay. C6 cells were incubated for 24 h with the sensitizer and were not irradiated

Colony survival after radiotherapy. The effect of radiotherapy was characterized by the dose–response relationship of cytotoxicity measured as a reduction of colony viability. The C6 glioma cells were irradiated with gamma rays using doses ranging from 0 to 8 Gy. Figure 3 shows the colony survival data for C6 cells exposed to gamma rays. The control plating efficiency of unirradiated cells was nearly 70% in the exponentially growing phase. The cytotoxicity was expressed as the percentage of colony survival relative to an untreated control. A cytotoxic dose inducing a reduction in colony survival by 20% was achieved at 2 Gy and by 50% at 4 Gy.

Colony survival after radiosensitized treatment. The colony-forming efficiency assay was used to assess the effect of sequential treatment of C6 cells with sensitizers followed by gamma irradiation. Clonogenic survival curves after irradiation with different concentrations of HpD and mTHPC are shown in Fig. 4. Different degrees of growth

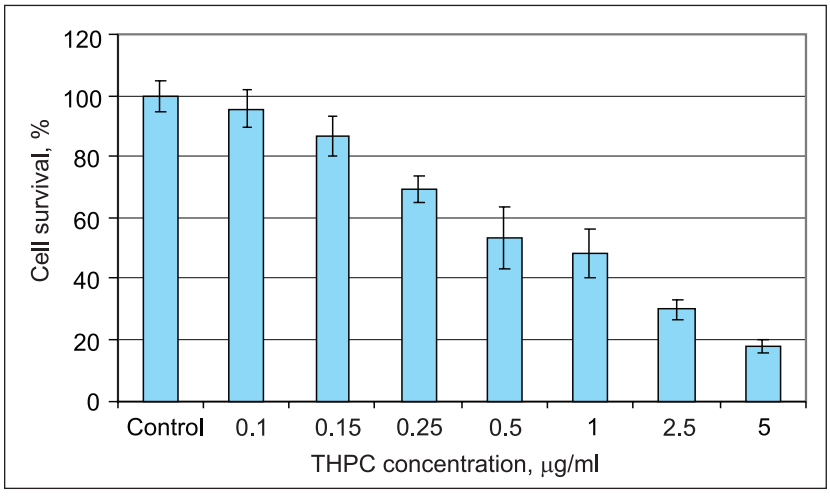

Fig. 2. Dark toxicity of mTHPC to C6 cells as determined by CV assay. C6 cells were incubated for 18 h with the sensitizer and were not irradiated

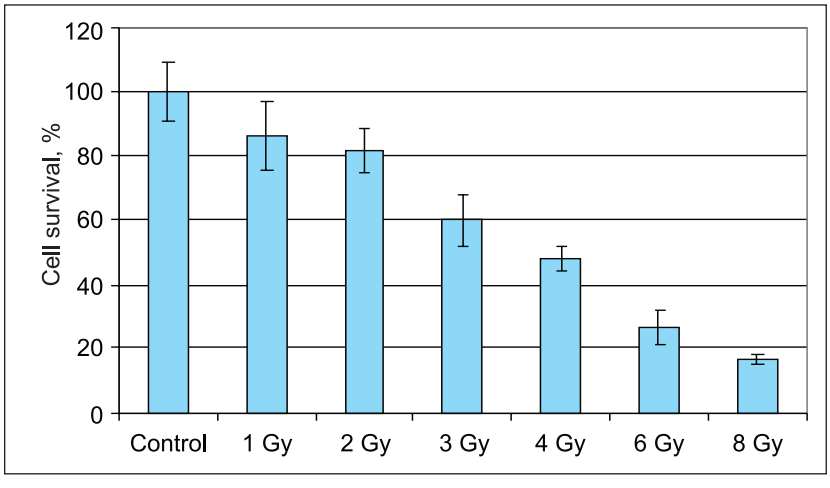

Fig. 3. Effect of gama-rays on the clonogenicity of C6 cells

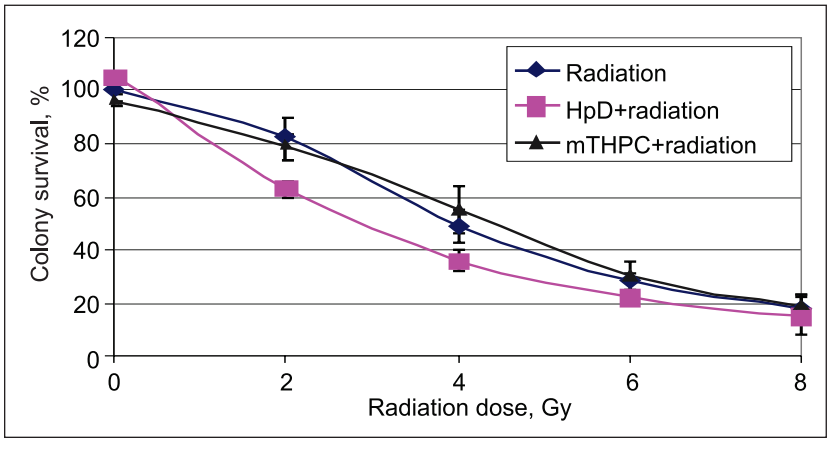

Fig. 4. Effect of gama-rays with porphyrins on the clonogenicity of C6 cells

inhibition were observed when radiation alone and HpD plus gamma rays were applied to C6 cells. The treatment of cells with HpD (1 μg/ml) for 24 hours before exposure to different doses of gamma-radiation resulted in a significant and dose-dependent decline in cell survival as compared with irradiation alone. HpD at this concentration is capable of inhibiting C6 glioma cell growth and acts as a radiosensitizer.

mTHPC at a concentration as used here (0.1 μg/ml) has no significant influence on the sensitivity of the C6 cells to gamma-radiation. Because there was no difference in survival between the two groups (with and without drug), it can be concluded that mTHPC does not act as a radiosensitizer.

DISCUSSION

Radiation or a combination of radiation and chemotherapy with temozolomide is still the most effective modality, after surgery, for the treatment of the majority of brain tumours (1, 2, 24). Radiotherapy is a treatment that has been consistently shown to extend survival after surgery, but most malignant gliomas show a resistance to radiation at dosages tolerated by normal brain tissue (5). Radioresistance of glioma cells may be partly due to the inability of therapeutic doses of ionizing radiation to induce apoptosis in glioma cells; necrosis is the primary mode of cell death after gamma irradiation (25). Glioblastoma multiforme is the most aggressive type of malignant gliomas and one of the highly angiogenesis-dependent tumours. Furthermore, most patients experience local recurrence and survive for less than a year after receiving therapy, due to the high proliferative and invasive potency of the remaining cells (3). An important goal in the management of malignant gliomas is, therefore, to increase cellular sensitivity to radiotherapy (5).

The toxicity of radiation to living tissues was discovered soon after the discovery of radioactivity itself, and this toxicity is the basis for cancer therapy with radiation. Although this mode of therapy is often effective, its success is far from assured. One major difficulty in the implementation of radiotherapy is that normal tissues are also sensitive to killing by radiation so that treatment is often limited by the tolerance of normal tissues to radiation. Thus, the methods that sensitize tumour cells while sparing normal tissues could potentially lead to a greater success of radiation as a therapy. Optimal sensitization to radiation requires that the tumor be targeted while the normal tissue is spared (26).

Two main factors – the local tumour control probability and normal tissue complication rate, which both result in the therapeutic ratio – must always be considered when using a radiosensitizing agent. Most of the presently known and routinely used radiosensitizing agents have a poor selectivity and are not tumour-specific. The use of these compounds may also result in severe side effects due to their inherent toxicity (13).

Photodynamic therapy using porphyrins as tumour localizing and photosensitizing drugs emerged as a promising alternative therapy for the treatment of early and localized tumours. However, the penetration depth of the laser light through the tissue is still less than 1 cm. So far, thick tumours and tumours located deep under the skin or in body tissues cannot be treated by this method because of a limited penetration of light in such tissues (27). It is necessary to develop more effective, higher oncotropic sensitizers and excitation devices with a long-range energy transfer capability (28).

It has been reported that porphyrins can also act as radiosensitizers under certain conditions (13, 16, 18, 19, 23, 28, 29). The mechanism of this radiosensitizing effect is not completely understood (30). *In vitro* data, however, support the hypothesis that the radiosensitizing action involves ROS generation (29) in addition to a potential impairment of repair mechanisms after a sublethal damage by ionizing radiation (30). When ionizing radiation interacts with a medium doped with a porphyrin, it has been proposed that its radiosensitivity is connected with the oligomers that can efficiently interact with some cytotoxic transient intermediates such as hydroxyl radicals which are known to be generated as a result of the primary interaction of ionising radiation with water. In this connection, the porphyrin acts as a radiation amplification factor by minimizing the possible onset of the repair processes which often limit the radio-induced cell damage (31). To date, a hypothesis has been proposed that ionising radiation activates porphyrin derivatives, resulting in a yield of red radio luminescence (29, 32).

In our study, we have shown that a combination of a hematoporphyrin derivative with gamma-radiation results in a significantly lower C6 glioma cell survival in clonogenic assays as compared with radiation alone. Our results demonstrate that C6 cells *in vitro* are radiosensitized by HpD. They are also in line with our other *in vivo* studies of rat glioma tumours (23).

CONCLUSIONS

The results of this study have shown that mTHPC (0.1 μg/ml) does not act as a radiosensitizer, but HpD can act, under certain conditions, as an effective tumour radiosensitizer. These findings suggest that HpD is a potential agent in combination with radiation therapy of malignant gliomas.

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Laimutė Rutkovskienė, Laima Plėšnienė, Daiva Sendiulienė, Alfonsas Stančius, Jurgita Liutkevičiūtė-Navickienė

ŽIURKIŲ C6 GLIOMOS LĄSTELIŲ ĮJAUTRINIMAS PORFIRINAIS PRIEŠ JONIZUOJANČIĄJĄ SPINDULIUOTĘ

Santrauka

Tikslas. Radiosensibilizatoriai (RS) naudojami onkologijoje siekiant padidinti radioterapijos efektyvumą. Fotodinaminėje terapijoje naudojami porfirinai selektyviai kaupiasi navikuose ir tuo skiriasi nuo kitų RS. Šio darbo tikslas – nustatyti, ar hematoporfirino darinys (HpD) arba temoporfinas (mTHPC) su jonizuojančiąja spinduliuote gali mažinti navikinių ląstelių išgyvenamumą.

Medžiagos ir metodai. Atlikti HpD (0–50 μg/ml) ir mTHPC (0–5 μg/ml) toksiškumo žiurkių C6 gliomos ląstelėms tamsoje eksperimentai. Tiriant radiosensibilizacijos efektus C6 ląstelės buvo inkubuotos su atitinkamos koncentracijos sensibilizatoriais (HpD – 1 μg/ml, mTHPC – 0,1 μg/ml) ir apšvitintos gama spinduliais (0–8 Gy). Ląstelių išgyvenamumas buvo tirtas klonogeninės analizės metodu.

Rezultatai. Nustatyta, kad atrinktos sensibilizatorių koncentracijos (HpD – 1 μg/ml, mTHPC – 0,1 μg/ml) nėra toksiškos C6 ląstelėms tamsoje. 2 Gy gama spindulių dozė be sensibilizatoriaus mažino gliomos kolonijų išgyvenamumą 20 %, 4 Gy dozė – 50 %. C6 ląstelių išgyvenamumo mažinimas, paveikus HpD ir gama spinduliais, buvo statistiškai patikimas, palyginus su kontrolinių, vien tik gama spinduliais paveiktų, ląstelių išgyvenamumu. mTHPC atveju skirtumo tarp kontrolinės ir tiriamosios grupių nenustatyta.

Išvados. Šio tyrimo rezultai rodo, kad HpD ir jonizuojančiosios spinduliuotės derinys tam tikromis sąlygomis statistiškai patikimai mažina C6 gliomos ląstelių išgyvenamumą, tačiau pasirinktos koncentracijos mTHPC (0,1 μg/ml) neveikia kaip radiosensibilizatorius.

Raktažodžiai: C6 gliomos ląstelės, radiosensibilizacija, porfirinai, klonogeninė analizė