Original article

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Effects of photodynamic exposure using chlorine E6 on U251 glioblastoma cell line *in vitro*

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Objective: to study the effect of photodynamic exposure with the use of chlorine E6 in cell cultures of the standardized human glioblastoma (GB) cell line U251 under different modes of laser irradiation (LI) *in vitro*.

Materials and methods. Groups of cell cultures of the U251 line were formed, depending on conditions of cultivation and exogenous influence: 1) control – cultivated in a standard nutrient medium (MEM with L-glutamine, 1 mml sodium pyruvate, 10% fetal calf serum) and experimental: 2) cultivated under conditions of adding a photosensitizer chlorine E6 (1.0, 2.0 and 3.0 μg/ml); 3) cultured in a nutrient medium without adding chlorine E6 and subjected to LI (intensity in the range 0.4–0.6 W, dose in the range 25–90 J/cm^2, continuous or pulse mode); 4) cultivated under the conditions of adding chlorine E6 and subsequent exposure to LI in the specified modes. Intravital dynamic observation with photo-registration (fluorescence and light microscopy, survey staining methods, intravital staining with a vital dye (0.2% trypan blue solution), morphometric studies (mitotic index, numerical density of viable cells) were carried out.

Results. Cell cultures of the human GB U251 line are characterized by the formation of peculiar intercellular connections (reticular histoarchitectonics) of tumor cells with high polymorphism and proliferation activity. Chlorine E6 is incorporated into the cytoplasm of U251 cells with preservation of fluorescence intensity for 72 hours (observation period). The fluorescence intensity of chlorine E6, incorporated by non-tumorally transformed cells of the rat fetal brain (E14-16), is much weaker. Under the influence of chlorine E6 (1.0, 2.0 and 3.0 μg/ml), cytodestructive processes in U251 cell culture increase in a dose-dependent manner with a progressive loss of viability and a decrease of mitotic index. After exposure to LI in the studied regimes the viability of U251 cells decreases in a dose-dependent manner already 1 h after exposure, with a further decrease after 24 h (the most significant (~30%) – at doses of LI 75–90 J/cm^2 in the pulse mode). Under the combined exposure of chlorine E6 (2.0 μg/ml) and LI, the viability of U251 cells decreases in a dose-dependent manner already 1 hour after exposure (by 4.5–10.0 times), the most significant (~80%) – at doses of LI 75–90 J/cm^2 in pulse mode. After 24 h of observation under all modes of combined exposure of chlorine E6 and LI, viable cells in U251 cultures were not detected**.**

Conclusions. Sufficient effectiveness of the cytodestructive effect of chlorine E6 (2.0 μg/ml, preincubation for 6–24 h) and the lowest studied dose of LI (25 J/cm^2) in the pulse mode in the cell culture of human GB U251 line was established. The use of vital dye provides an opportunity to record cytotoxic effects in the culture of U251 tumor cells at an early stage (within 1 h after exposure to chlorine E6 and LI).

Key words: *laser irradiation; chlorine E6; human glioblastoma U251 cell culture; cytodestructive effects; mitotic index; cell viability*

Glioblastoma (GB) is the most common malignancy among primary brain tumours and other central nervous system (CNS) tumours [1,2], which has been classified as IDH wildtype, according to the WHO approved updated edition CNS tumor classification (2021) [3]. The use of combined treatment (surgical removal, radiation and chemotherapy) - the modern standard of treatment of GB [4] significantly increases overall survival of patients with primary GB [5]. However, GB remains one of the most treatment-resistant primary CNS malignant tumours, with a progressive progredient course. Most cases of continued growth/recurrence occur

in the marginal (perifocal) area of the surgical cavity due to invasive properties of gliomas and the presence of residual tumour cells [6], since the nature of GB spread and the extent of surgical resection are difficult to definitively assess when using classical surgical approaches and intraoperative imaging techniques. A number of techniques, including photodynamic (photodynamic diagnostics, fluorescence-guided surgery and adjuvant photodynamic therapy (PDT)) have been proposed to address this problem [6, 7].

Photodynamic therapy is a technique that allows simultaneous visual identification of the spread (borders) of tumour tissue and its selective destruction. It involves two stages: the introduction of a photosensitive agent (photosensitizer (PS)) and its activation by light rays to target tumor cells. After administration (intravenous, intraperitoneal, local or oral), PSs pass the blood-brain barrier unempeded and accumulate selectively in tumour tissue compared to normal brain tissue (the ratio is 50:1 and 3:1, respectively) [8].

The PDT method is based on cytotoxic effects caused by a cascade of molecular events. Activation of PS, selectively accumulated in neoplasm cells, by light rays of the appropriate wavelength [9, 10] generates singlet molecular oxygen and reactive oxygen species that trigger photochemical reactions in tumour cells with the destruction of their macromolecules, which leads to the death of tumour cells (due to necrosis, apoptosis, autophagy and other variants of programmed cell death) and activates the mechanisms of both direct cytodestructive and mediated immunomodulatory antitumour effects, involving the links of innate and acquired immune defence [10–13].

Generation 1, 2, and 3 PSs have been developed, but the latter not yet approved for clinical use. *In vitro* and *in vivo* experimental studies have confirmed the efficacy of PDT of brain tumours using 1st and 2nd generation PSs, both alone [14–16] and in combination with radiotherapy, chemotherapy, sonodynamic therapy or epidermal growth factor receptor (EGFR) inhibitors [17–21]. The results of clinical trials (most are uncontrolled, phases I/II) using 1st and 2nd generation PSs (HpD, talaporfin sodium, porfimer sodium, 5-ALA) [22–27], despite difficulties with analysis and evaluation of treatment efficacy due to significant differences in the implementation schemes, testify that PDT contributes to the increase of tumour destruction zone during surgery and survival rate, improves the quality of life of patients with malignant gliomas, and has low systemic toxicity.

The efficiency of the photodynamic action on a sensitized cell is determined by the intracellular concentration of PS, its localization in cellular compartments, photochemical activity, as well as laser irradiation (LI) dose. Therefore, the development of experimental models of PDT to optimize doses and regimes of LI is relevant. Chlorine E6 PDT has been previously reported to be promising in C6 and 101.8 rat glioma models *in vivo* [28]. Chlorine E6 is a 2nd generation PS ***(Fig. 1)***.

The analysis of scientific resources to develop adequate methodological approaches proves the relevance and feasibility of preclinical studies of PDT effect on tumour growth models *in vitro*.

Objective: to study the effect of photodynamic exposure using chlorine E6 in cell cultures of the standardized human glioblastoma (GB) cell line U251 under different regimens of laser irradiation (LI) *in vitro*.

Materials and methods

The study was carried out on human GB cell cultures of the U251 line. Cryopreserved cell samples (provided by the "Cell Bank of Human and Animal Tissue Lines", R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv) were thawed in a water bath for 30 min at 38 °C, suspended in nutrient medium (MEM with L-glutamine ("Biowest", France), 1 mmol sodium pyruvate, 10% fetal calf serum ("Biowest", France)), and were placed in culture plastic vials (92 × 51 × 29 cm, "TPP Techno Plastic Products AG", Switzerland) in the amount of $0{,}1 \cdot 10^6$ cells/8 ml of growth medium. Cultures were kept in a CO_2 incubator ("Nuve", Turkey) under standard conditions (95% humidity, 37 °C, 5% CO_2) for 3 passages. The growth medium was changed every three days. Dynamic observation with step-by-step photomicrograph registration was performed using an inverted microscope "Nikon S-100" (Japan). Cells from the 4th passage were suspended in the growth medium and transferred in amount of 2×10^6 in plastic Petri dishes (d – 35 mm, "SPL", South Korea) on coverslips precoated with polyethyleneimine (Sigma-

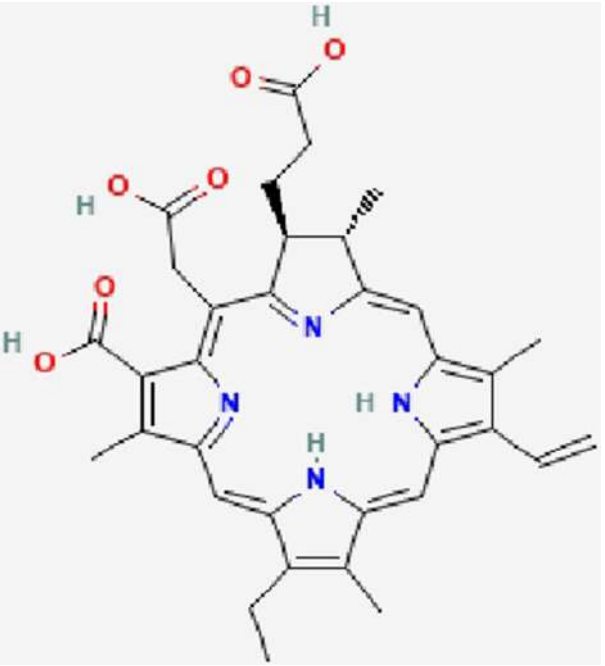

Fig. 1. Chemical structure of chlorine E6 (molecular formula $C_{34}H_{36}N_4O_6$, other known names are photochlorine, Photolon, Phytochlorin, Phytochlorine) [29]

This article contains some figures that are displayed in color online but in black and white in the print edition

Aldrich, GmbH, Germany), added nutrient medium (2 ml) and cultured until a monolayer was reached 75–80%.

To investigate the direct effects of chlorine E6, a photosensitizing agent (at a concentration of 1.0, 2.0, and 3.0 μg/ml) was added to the U251 cell line cultures with the formed monolayer, then the cultures were kept in a CO_2 incubator ("Nuve", Turkey) and dynamic observation with photomicrograph registration was performed using an inverted microscope "Nikon S-100" for 48–72 hours. The fluorescence study was carried out using an Axiophot microscope ("Opton", Germany) using fluorescent filters (λ–500–680 nm). To compare the selectivity of PS incorporation into cells of the U251 line, chlorine E6 was also added to cultures of rat fetal brain neurogenic cells obtained according to the protocol [30].

To study the direct effects of LI exposure, cultures of U251 cell line with a formed monolayer were exposed to LI using the "LIKA-surgeon" equipment ("Photonika-Plus", Ukraine). For this purpose, Petri dishes with cultures were placed under a vertical fiber optic laser output (h – 5 cm) and irradiated with uniform coverage of the monolayer area by light rays (λ – 660 nm) under different modes (intensity range – 0.4–0.6 W, dose – 25–90 J/cm^2, continuous or pulse mode). Cultures were then kept in a CO_2 incubator ("Nuve", Turkey) and dynamic observation with photomicrograph registration using an inverted microscope "Nikon S-100" for 24 h and fluorescence study was performed.

For the study of combined effect of chlorine E6 and LI (PDT), photosensitizing agent (at the concentration of 1.0 and 2.0 μg/ml) was added to the U251 cell line cultures with a formed monolayer and kept in a CO_2 incubator ("Nuve", Turkey) for 4 or 24 hours. After that, the dishes with cultures were placed under a vertical fiber optic laser output (h – 5 cm) and exposed to light rays (λ – 660 nm) in different modes, as indicated above. Then the cultures were kept in a CO_2 incubator ("Nuve", Turkey) and dynamic observation with photomicrograph registration using an inverted microscope "Nikon S-100" for 24 h and fluorescence study was performed.

To determine intravital cytotoxic effect of chlorine E6 and LI, cultures of U251 cell line with a formed monolayer were exposed to different research conditions (described above) with the addition of a vital dye (0.2% trypan blue solution ("Merck", Germany)) into the culture medium and intravital observation of the viability of U251 cell line cultures using an inverted microscope "Nikon S-100" was carried out.

For further analysis, groups of cell cultures of the U251 line were formed depending on conditions of cultivation and exogenous influence: 1) control - cultivated in a standard growth medium (MEM with L-glutamine, 1 mmol of sodium pyruvate, 10% fetal calf serum) and experimental: 2) cultivated under the conditions of adding PS chlorine E6 (1.0, 2.0 and 3.0 μg/ml), 3) cultured in a nutrient medium without adding PS and subjected to LI (intensity range - 0.4–0.6 W, dose - 25–90 J/cm^2, continuous or pulse mode), 4) cultured under conditions of adding chlorine E6 and subjected to LI (intensity range - 0.4–0.6 W, dose - 25–90 J/cm^2, continuous or pulse mode).

Control and experimental cell cultures were fixed in 10% neutral formalin ("Bio-Optica", Italy) and stained with hematoxylin and eosin according to Carracci. Microscopic examination and photoregistration of culture preparations was performed using a light-optical photomicroscope "Nikon Eclipse E200" (Japan). In each preparation, the peculiarities of the structure of the experimental cultures compared to the control ones were analyzed. The cellular composition of the growth zone of the cultures was evaluated by morphological characteristics of the structure of tumor cells and their ability to form the spatial histoarchitectonics of the monolayer. The shape of tumor cells, presence and branching of processes, structure and shape of nuclei, the nature of chromatin distribution, and the features of intercellular interactions were analyzed.

Quantitative studies of control and experimental cultures were performed in 10 representative fields of view with a standard measuring scale (object micrometer). Morphometric analysis was carried out by processing digital images of cultures in 10 randomly selected fields of view for each sample at the same magnification (×400) using ImageView software (PRC, 2020), determining on the test area the number of viable cells, total number of cells, number of cells in mitotic division state. The mitotic index (MI, %) was calculated as the proportion of cells with the presence of mitoses from the total number of cells.

Statistical analysis of the obtained data was performed using Statistica 8.0 statistical software package, StatSoft, Inc. (2007). Nonparametric methods of variation statistics were used (Kruskel-Wallis ANOVA on ranks for multiple comparison of several independent groups, Mann-Whitney U-test for pairwise comparison of independent groups, Wilcoxon test for pairwise comparison of dependent groups (in observational dynamics). Normality of data distribution was determined by the Shapiro–Wilk test. Data are given as (M±m), where M is the average value, m is the standard deviation from the average value. Differences were considered as statistically significant at $p < 0.05$.

Results and discussion

Cell cultures of GB, which belongs to the most malignant brain gliomas, are characterized by metabolic and genetic heterogeneity, significant cell polymorphism and high mitotic activity, the manifestations of which *in vitro* correlate with the degree of tumour tissue anaplasia *in vivo*. Under cultivation conditions, even the most malignant polymorphic forms of GB show the ability to differentiate in a genetically programmed astrocytic phenotype in some tumour cells. However, the significant heterogeneity of the glioma cell population necessitates the use of standardized experimental conditions of tumor growth models. We have used as a model of tumor growth human GB cell cultures of the U251 line, a cell line derived by explantation from a malignant human brain GB tumour, the cell type of which is defined as pleomorphic/astrocytoid [31].

Characterization of human GB cultures of the U251 cell line. Within 24 h after cell passage, the formation of chains and dense monolayer cell conglomerates was observed in the cultures, between which there were tumour cells without clear signs of differentiation - with narrow cytoplasm and moderate

nuclear polymorphism, as well as cells with formed processes ***(Fig. 2, A)***. On the 5th day, increased growth and proliferation of tumor cells monolayer was detected. The cells are large, with clear contours, distinct cytoplasm, a large nucleus, unipolar, triangular, diamond-shaped, polygonal in shape with elongated processes ***(Fig. 2, B)***. In more rarefied parts of the growth zone, the formation of peculiar intercellular connections with the formation of reticular histoarchitectonics was noted. Thus, some GB cells of the U251 line showed signs of differentiation with the formation of characteristic reticular structures. Also, in the marginal areas of the culture growth zone, the predominance of cells with a broad cytoplasm, similar to an epithelioid monolayer, was observed. Upon reaching the confluent stage of growth, characteristic reticular growths of densely packed tumour cells with high polymorphism and short dendritic processes were observed in the cultures. On histological preparations in the growth zone of cultures in the field of view (×400), 2-3 tumor cells in a state of mitotic division were detected (MI on average was (2.26±0.11)% after 72 h of cultivation), indicating high proliferation activity ***(Fig. 2, C)***.

Effects of chlorine E6 in human GB cell culture of the U251 line. According to the fluorescence study, in experimental cell cultures of the U251 line, PS chlorine E6 accumulated predominantly in the cytoplasm of tumour cells. The fluorescence intensity of the incorporated PS was maintained throughout the observation period (72 h, ***Fig. 3, A***).

In contrast to cells of the U251 line, the fluorescence intensity of PS incorporated by non-tumorally transformed rat fetal brain cells (E14, used as the intracellular PS incorporation control) was much weaker ***(Fig. 3, C)***, which is consistent with the data on higher intensity of selective accumulation of PS by tumour tissue [8].

Using different concentrations of PS (1.0, 2.0, and 3.0 μg/ml) showed that cytodestructive processes increase in a dose-dependent manner in the human GB cells culture of the U251 line when exposed to chlorine E6 for 24–48 h ***(Fig. 3, B)***.

When exposed to chlorine E6 at a concentration of 1.0 μg/ml after 48 h in cell cultures of the U251 line, rarefaction of the growth zone with the appearance of large lacunae in the cell monolayer was observed. Against the background of intact cells, diffusely arranged dystrophic or necrobiotically altered tumour cells with reduced processes with rounded cytoplasm with signs of lipid and hydropic cytoplasm distrophy and hyperchromic nuclei were found ***(Fig. 4, A)***. Some tumour cells have turned into shadow cells and "naked" nuclei. The number of mitoses decreased to 1-2 in the field of view (MI – (1.14±0.09) %). Abnormal forms of mitoses were observed [32] among figures of mitotic division of tumour cells ***(Fig. 4, B)***.

When exposed to chlorine E6 at a concentration of 2.0 μg/ml in cell cultures of the U251 line after 6 h, a rarefaction of the density of cell monolayer was observed due to retraction and reduction of the processes of damaged cells in the growth area. Cells with signs of dystrophy of varying severity of expressiveness were found in the preserved separate areas of the reticular structures. These changes increased during the next 24–48 h: the content of tumour cells with cytological features of dystrophy and necrobiosis (pyknotic nuclei and vacuolated cytoplasm, ***Fig. 4, C***) increased, up to 1 mitosis was observed per few fields of view (MI – (0.26± 0.08%), ***Fig. 4, D***).

Increasing the duration of incubation of cells of the U251 line with chlorine E6 from 6 to 48 h resulted in progressive loss of tumour cells viability by an average of 20% ($p=9\cdot10^{-4}$ compared to control, Mann-Whitney U-test; p=0.04 compared to previous incubation period, Wilcoxon test; ***Fig. 5, A***).

After 72 h of cultivation in cell cultures of the U251 line with PS added at a concentration of 2.0 μg/ml, thinning of the growth zone, the appearance of degenerated and necrobiotically altered tumour cells with a decrease in processes, and shadow cells formation occured. The value of MI decreased from (2.26±0.11)% (control) to (0.15±0.06)% ($p=3,3\cdot10^{-5}$, Mann-Whitney U-test).

Consequently, the results of evaluating the effect of chlorine E6 demonstrate a dose-dependent cytotoxic effect on human GB tumour cells of the U251 line, which is enhanced with increasing incubation time of cultures with this drug.

Effects of LI at different regimes in the human GB cells culture of the U251 line.

When exposed to LI (intensity – 0.4 W, dose – 50 J/cm^2, continuous mode) in certain areas of the growth

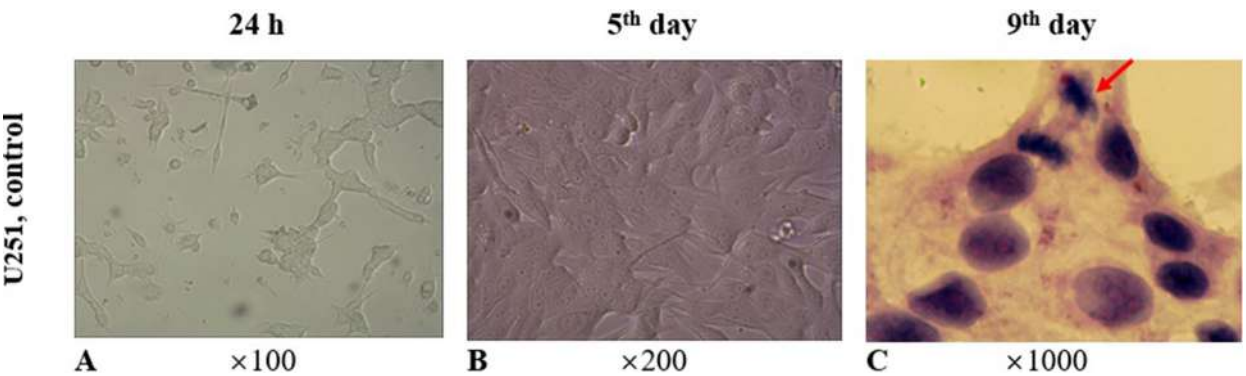

Fig. 2. Photomicrograph of human glioblastoma cell cultures of the U251 line cultivated in a standard nutrient medium (control). Light microscopy, unstained culture (A, B); hematoxylin and eosin staining (C). The arrow indicates a cell in the stage of mitosis (anaphase)

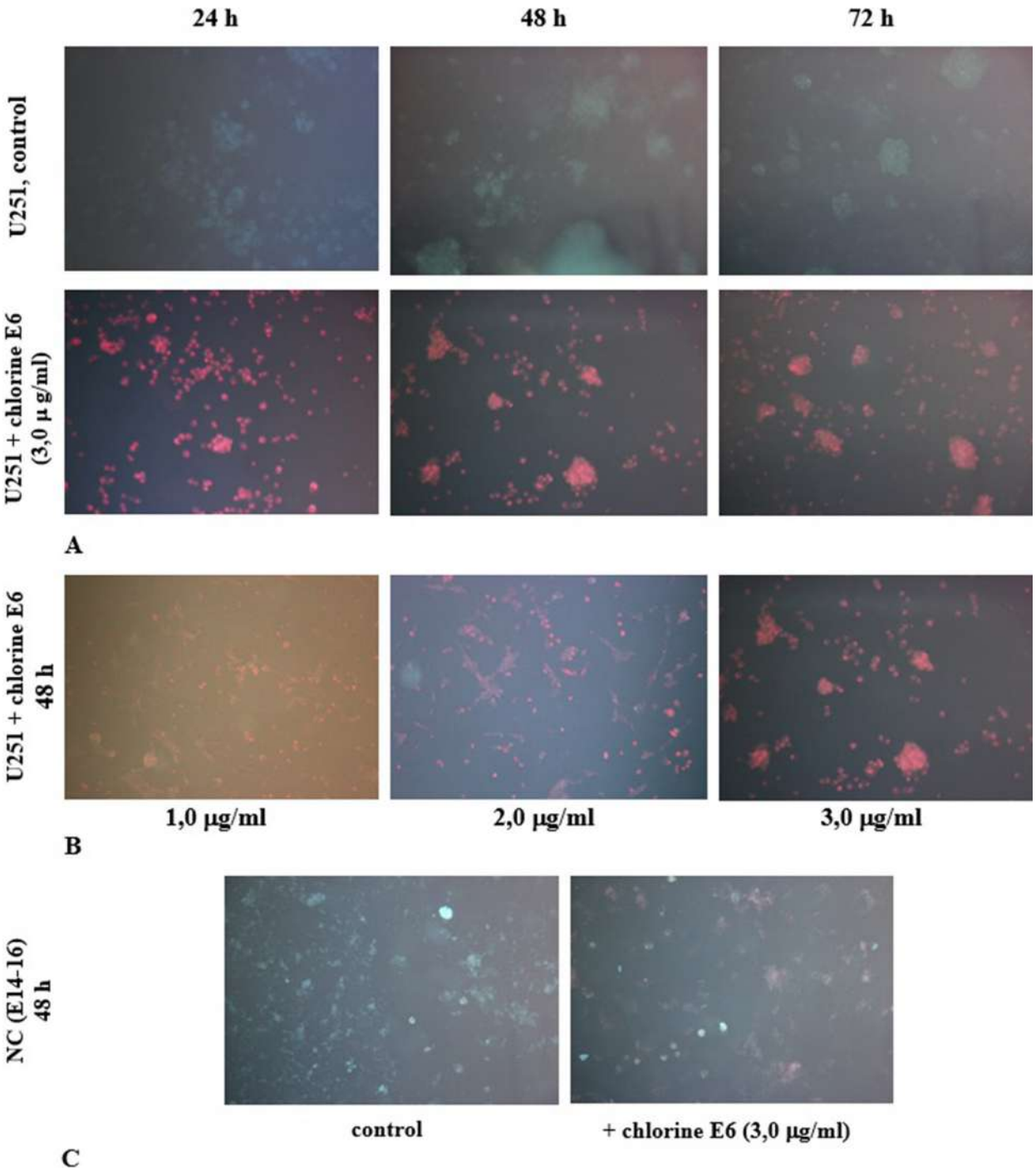

Fig. 3. Photomicrographs of cultures cultivated in a standard nutrient medium and after adding chlorine E6: A, B – human glioblastoma cells of the U251 line at different incubation times (A) and different concentrations of chlorine E6 (B); C – neurogenic cells (NC) of rat fetal brain tissue (E14), control of chlorine E6 incorporation. Fluorescence microscopy. ×200

zone of GB cultures of the U251 line, rarefaction of the cell monolayer due to desquamation of degenerated cells and cells with clear signs of dystrophy and necrobiosis (reduced processes, rounded cytoplasm, hyperchromic nuclei) were detected. In the growth zone, cytoplasmic vacuolization, appearance of significant amount of apoptotic bodies, which indicates the apoptotic type of tumor cell death, signs of nuclear polymorphism, were recorded in the cells. Some tumour cells turned into shadow cells, "naked" nuclei, but in some areas of the growth zone of the cultures, mitotic activity of the cells was preserved. A similar picture is characteristic of the influence of LI with an intensity of 0.6 W, a dose of 25 J/cm^2, continuous mode ***(Fig. 6)***. Under the same conditions (0.6 W, 25 J/cm^2), but in the pulse mode, LI caused spontaneous death of part of the cells of the U251 line.

Increasing the LI dose up to 50 J/cm^2 in a continuous mode with a power of 0.6 W led to an increase in dystrophic and necrobiotic changes in the cells of

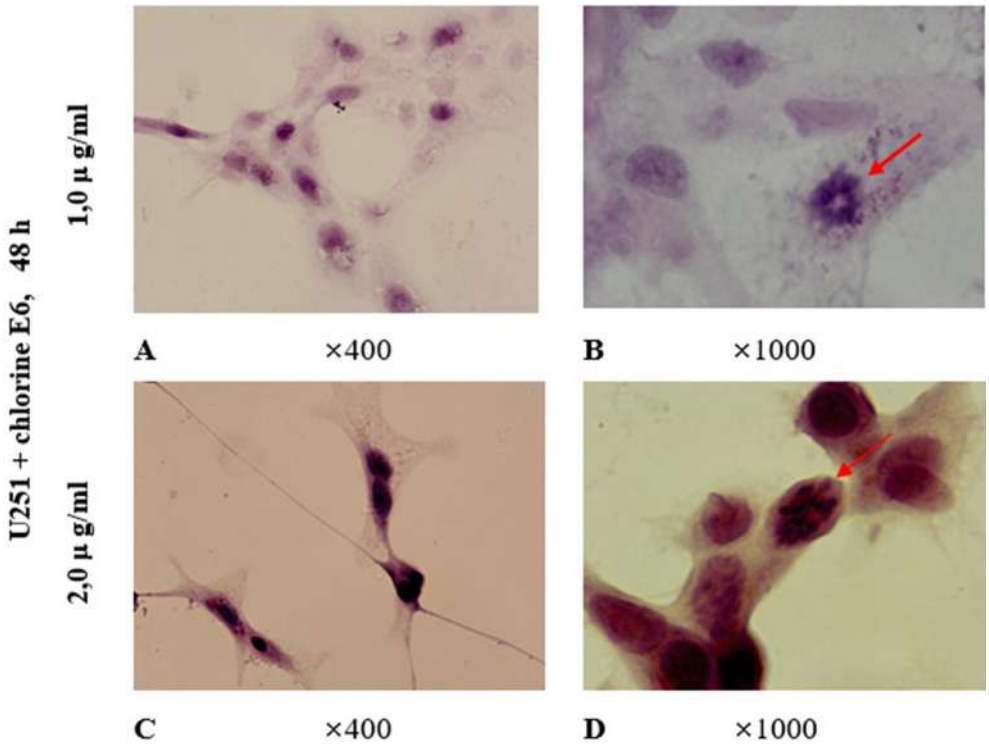

Fig. 4. Photomicrograph of cultures of human glioblastoma cells of the U251 line, cultivated in a standard nutrient medium and after adding chlorine E6 at different concentrations. Light microscopy. Staining with hematoxylin and eosin. Arrows indicate abnormal forms of mitoses (hollow metaphase (B), pulverization of chromosomes (D)

the U251 line, appearance of a significant number of apoptotic bodies. Under the same conditions (0.6 W, 50 J/cm^2), but in pulse mode, LI led to the intensification of destructive processes in the growth zone of cells of the U251 line ***(Fig. 6)***, necrobiotic processes of spontaneous death were characteristic for the majority cells in the growth zone.

After exposure to LI with a power of 0.6 W, doses of 75 and 90 J/cm^2, both in continuous and pulse mode, increased destruction of the growth zone and intensification of dystrophic and necrobiotic changes in cells were found ***(see Fig. 6)***. Chromatin coagulation with transformation into a sharply basophilic homogeneous mass (pyknosis) was observed in some cells, indicating a violation of nuclear membrane integrity. On cytological preparations in the preserved areas of growth zone an increased number of dystrophically changed cells with appearance of a large number of shadow cells and "naked nuclei" were detected.

Increasing the LI power to 1 W resulted in abrupt devastation of the growth zone with almost subtotal destruction of tumour cells. After LI in continuous mode with a power of 1 W, a dose of 50 J/cm^2, diffusely located individual tumour cells with processes or small clusters of such cells were observed in the growth zone. On cytological preparations an increase in the content of pathologically changed cells with signs of degeneration were registered.

Therefore, the results of the study with a morphometric evaluation of peculiarities of different modes of influence of LI on cultures of GB cells of the U251 line indicate a dose-dependent cytotoxic effect of its action. An increase in power from 0.4 to 1.0 W and the dose from 25 to 90 J/cm^2 in a continuous mode leads to destructive changes in the growth zone histoarchitectonics (from growth zone retraction with the formation of lacunae of various sizes at the lowest LI indicators to the gross destruction of the architecture of the monolayer and significant arrays of cellular devastation at the highest LI values). A decrease in mitotic activity of tumour cells and an increase in dystrophic-degenerative irreversible changes up to necrobiosis and necrosis with followed desquamation of dead cells from the adhesive surface were also noted. This effect is enhanced with the same characteristics of the LI in the pulse mode.

Study of the LI effect (λ – 660 nm, intensity – 0.6 W, dose – 25–90 J/cm^2, continuous or pulse mode) using intravital staining with the vital dye trypan blue (0.2%) showed that tumour cells viability in the culture cells of the U251 line, depending on the applied dose, starts decreasing as early as 1 h after exposure. The decrease continues after 24 h of observation ($p<0.03$ compared to control, Mann-Whitney U-test; see ***Fig. 5, B***). A definite dependence of the loss of viability of tumour cells on the radiation dose and its mode was found. The most significant decrease in viability (~30%) was recorded at a LI dose of 75–90 J/cm^2 in pulse mode ($p<0.003$ compared to the control, Mann-Whitney U-test; see ***Fig. 5, C***).

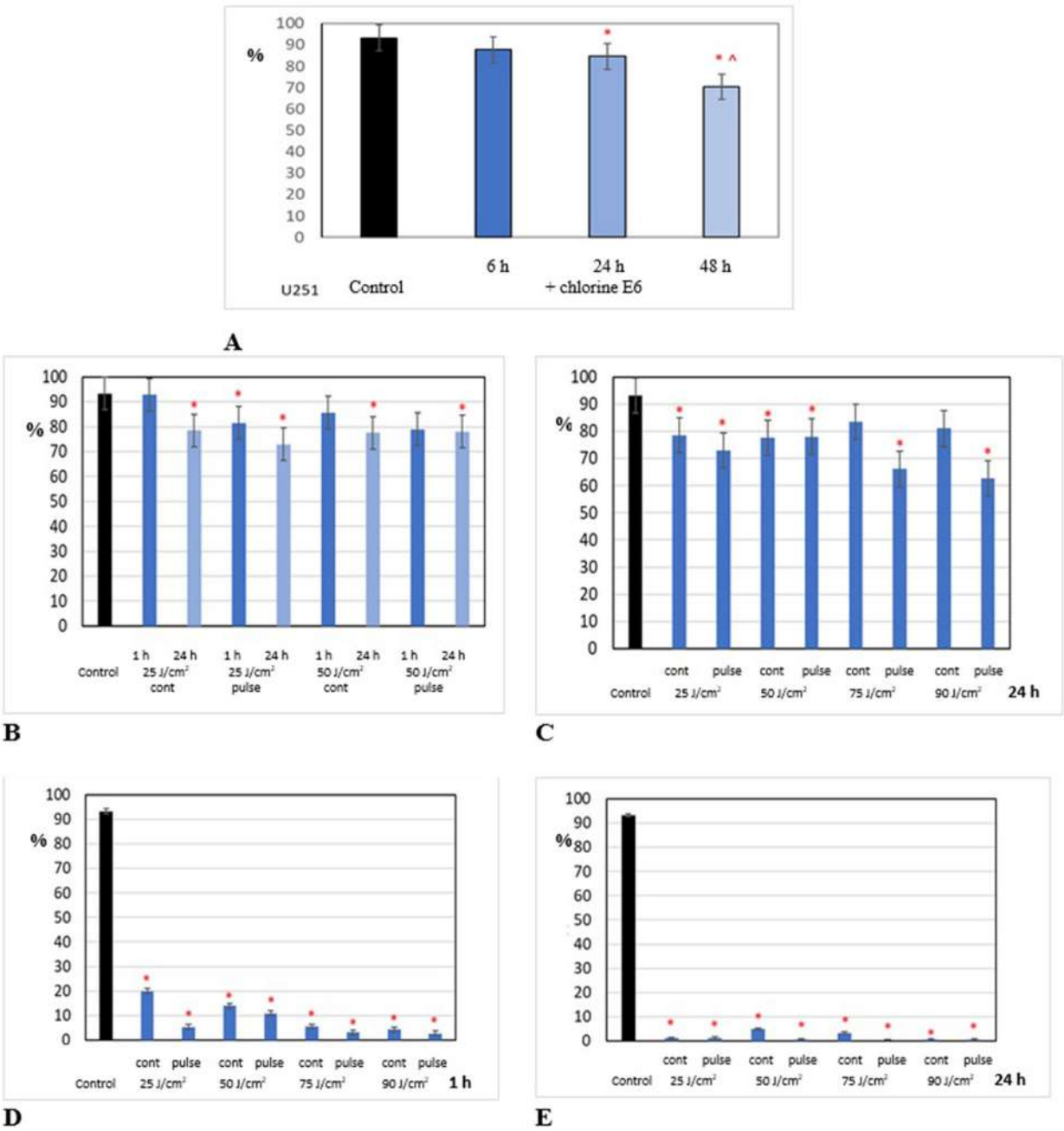

Fig. 5. Relative number of viable tumour cells in the human glioblastoma cells culture of the U251 line: A – depending on the incubation period with chlorine E6 (2.0 μg/ml); B, C – under the influence of different modes of laser irradiation (0.6 W); D, E – under the combined effect of chlorine E6 (2.0 μg/ml, preincubation for 6 h) and different modes of laser irradiation (0.6 W). * – $p<0.05$ compared to the control (Mann-Whitney U-test); ^ – $p=0.04$ compared to the previous incubation period (Wilcoxon test). Cont – continuous mode; pulse – pulse mode

The effects of the combined exposure to chlorine E6 and LI under different regimes in the human GB cells culture of the U251 line. In cultures of cells of the U251 line, after 24 h incubation with chlorine E6 (1.0 μg/ml) and subsequent LI (0.6 W, 75 J/cm^2, continuous and pulse mode), the cell population density decreased significantly, there were signs of subtotal atypicality (binuclear cells and cells with micronuclei), as well as cells at different stages of apoptosis (by indirect structural features). In the cells, vacuolated cytoplasm and nuclear abnormalities (atypical nuclear shape, karyorrhexis) were observed, as well as nuclei at different stages of cell death (from vacuolization of the central part of the nucleus to karyopyknosis, ***Fig. 7***). No mitotic activity was recorded.

Increasing the concentration of chlorine E6 to 2.0 μg/ml followed by LI with a gradual increase in power and dose resulted in even greater destructive changes in the cell monolayer.

Study of combined exposure of chlorine E6 (2.0 μg/ml, preincubation for 6–24 h and LI in different modes (λ – 660 nm, intensity – 0.6 W, dose – 25–90 J/cm^2, continuous

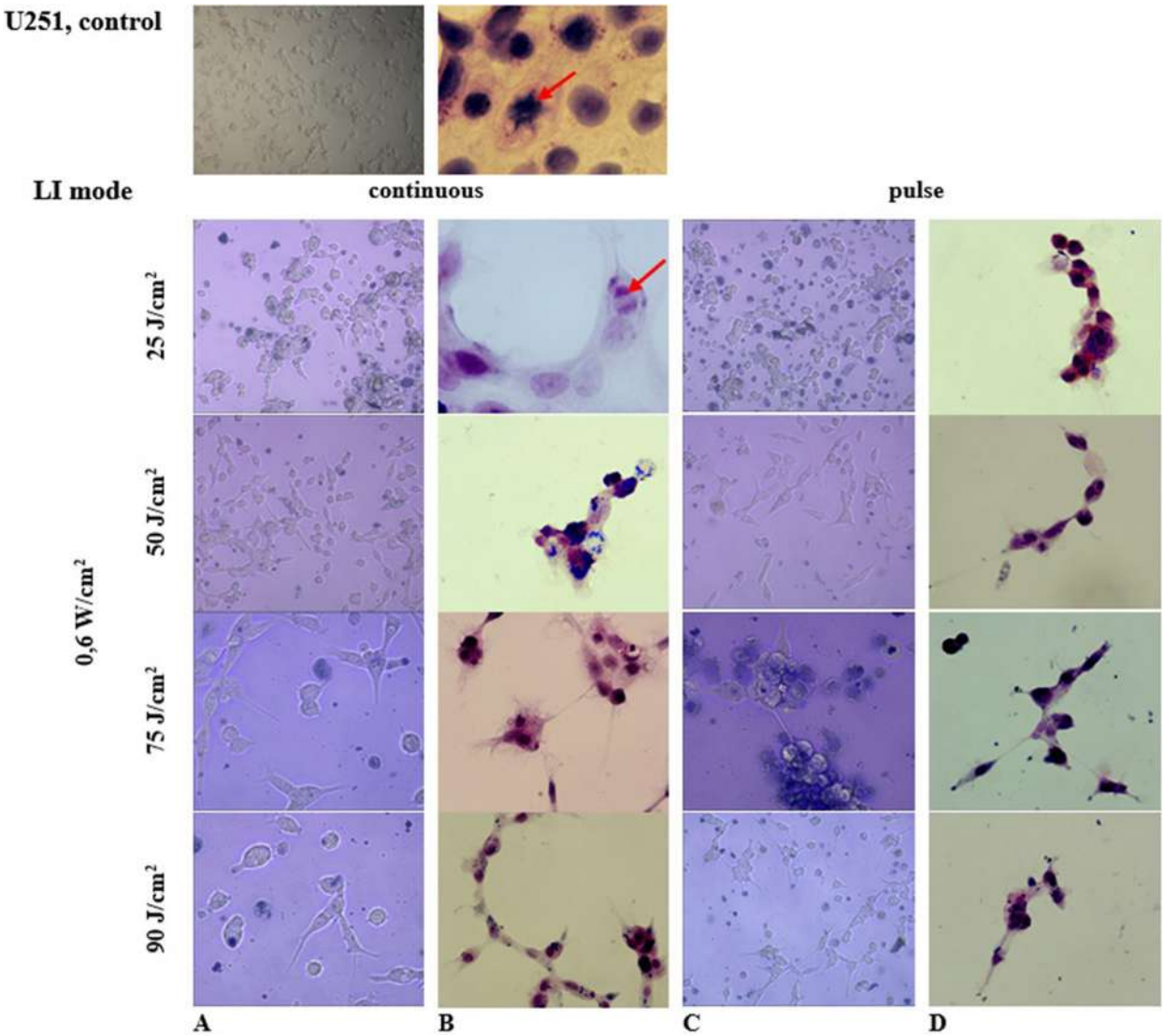

Fig. 6. Photomicrograph of human glioblastoma cell cultures of the U251 line when exposed to laser irradiation in different modes. Light microscopy. Staining with 0.2% trypan blue (A, C) and hematoxylin and eosin (B, D). Arrows indicate cells in the state of mitosis

or pulse mode) showed that the relative number of viable tumor cells in cell cultures of the U251 line, depending on the dose and mode applied, decreases already 1 h after exposure by 4.5–10.0 times ***(see Fig. 5, D)***. The greatest decrease in viability (~80%) was recorded at LI doses of 75–90 J/cm^2 in the pulse mode ($p<0.003$ compared to the control, Mann–Whitney U-test; ***see Fig. 5, D***). After 24 hours of observation under all modes of irradiation no viable cells were detected in cell cultures of the U251 line ($p<0.001$ compared to the control, Mann–Whitney U-test; ***see Fig. 5, E***), which gives grounds to conclude that the application of the lowest studied irradiation dose (25 J/ cm^2) in the pulse mode with preincubation of the cell culture with chlorine E6 for 6 hours is sufficiently effective.

Thus, as a result of morphological and morphometric study of cytotoxic effect of the photosensitizing agent (chlorine E6), exposure to LI (λ – 660 nm, intensity – 0.4–0.6 W, dose – 25–90 J/cm^2, continuous or pulse mode) and the combined effect of chlorine E6 and LI in the indicated modes, it was established that exposure to chlorine E6 for 24–48 hours dose-dependently increases cytodestructive (dystrophic-degenerative) processes in the culture of human GB cells of the U251 line, the combination of exposure to chlorine E6 and LI with an increase in dose leads to almost complete destruction of tumour cells in culture. The use of vital dye (0.2% trypan blue solution) enables early detection of cytotoxic effects at an early stage (within one hour after combined exposure to chlorine E6 and LI).

The method of intravital staining of tumour cells culture with a vital dye makes it possible to evaluate potential cytotoxic effects in a culture of tumour cells under the influence of studied experimental conditions, as well as other physical and chemical agents, which may form the basis for the theoretical substantiation for the development of efficient methods of rapid assessment of tumour cells sensitivity in a specific clinical case (individual sensitivity) to potential selective antiblastic factors in the treatment of neurooncological diseases.

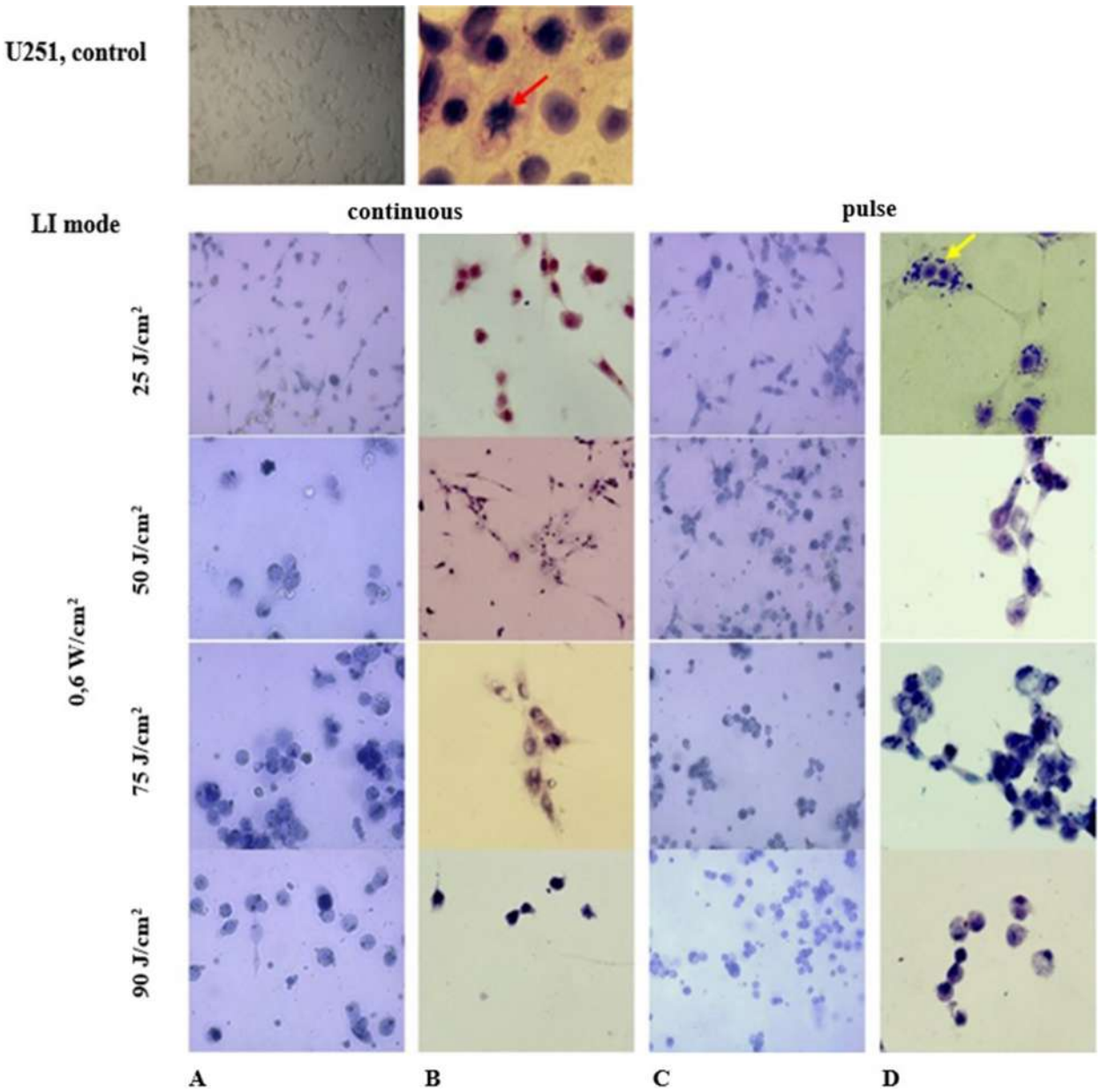

Fig. 7. Photomicrograph of human glioblastoma cell cultures of the U251 line under the combined effect of chlorine E6 (2.0 μg/ml) and laser irradiation in different modes. Light microscopy. Staining with 0.2% trypan blue (A, C) and hematoxylin and eosin (B, D). Arrows indicate a cell in the state of mitosis (red arrow) and a binuclear cell (yellow arrow)

Conclusions

As a result of the morphological and morphometric study in the culture of human GB cells of the U251 line, the cytodestructive effect of the combined use of chlorine E6 and LI was proven, and their effective doses and regimens were established.

1. Chlorine E6 is incorporated into the cytoplasm of GB cells of the U251 line and maintains the fluorescence intensity throughout the observation period (72 h).

2. Exposure to chlorine E6 (1.0, 2.0 and 3.0 μg/ml) promotes dose-dependent cytodestructive changes in the culture of human GB cells of the U251 line with a progressive loss of tumour cells viability and a decrease in the value of MI.

3. After exposure to LI (λ – 660 nm, intensity – 0.6 W, dose – 25–90 J/cm^2, continuous or pulse mode) the viability of tumour cells in the culture of human GB cells of the U251 line dose-dependently decreases 1 hour after exposure followed by a decrease after 24 h of observation. The most significant decrease in viability (~30%) was recorded for LI doses of 75–90 J/cm^2 in pulse mode.

4. Under conditions of combined exposure to chlorine E6 (2.0 μg/ml, preincubation for 6–24 h) and LI in different modes (λ – 660 nm, intensity – 0.6 W, dose – 25–90 J/cm^2 , continuous or pulse mode) the tumor cells viability in the culture of human GB cells of the U251 line decreases dose-dependently 1 h after exposure, the most significant (~80%) – at LI doses of

75–90 J/cm^2 in the pulse mode. After 24 h of observation after all irradiation modes, no viable cells were detected in cell cultures of the U251 line, that gives evidence for sufficient effectiveness of the lowest irradiation dose studied (25 J/cm^2) in the pulse mode at previous incubation of the cell culture with chlorine E6 for 6 hours.

5. Using vital dye (0.2% trypan blue solution) makes it possible to register cytotoxic effects at an early stage (within one hour after combined exposure to chlorine E6 and LI).

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Disclosure

Conflict of interest

The authors declare no conflict of interest.

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