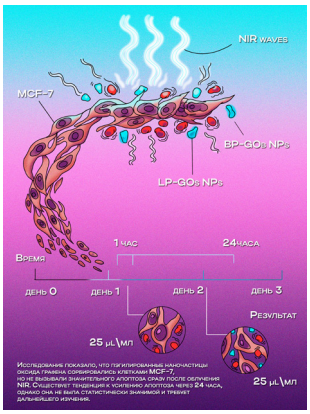
Original research article

EVALUATION OF THE EFFECTS OF GRAPHENE OXIDE NANOPARTICLES ON MCF-7 CELLS UNDER NIR IRRADIATION

M. D. Dolgikh[1, 2], M. S. Bochkova[1, 2], V. P. Timganova[2], S. A. Zamorina[1, 2]*



[1] Perm State National Research University, 614068, Perm, Russia
[2] Institute of Ecology and Genetics of Microorganisms, Ural Branch of the Russian Academy of Sciences, 620049, Perm, Russia

* Correspondence: mantissa7@mail.ru

Abstract: The development of novel approaches to induce apoptosis in tumour cells remains a key challenge in contemporary biomedicine. Given the exceptional thermal conductivity of graphene, we employed one of its derivatives — graphene oxide (GO) — to achieve localised heating of cells under near-infrared (NIR) irradiation *in vitro*. In this study, graphene oxide nanoparticles coated with either linear (LP-GOs, Ø184 ± 73 nm) or branched (BP-GOb, Ø1376 ± 48 nm) polyethylene glycol were used. The MCF-7 breast adenocarcinoma cell line served as the cancer model. Cells were cultured for 24 hours to allow monolayer formation, after which LP-GOs and BP-GOb nanoparticles were added at final concentrations of 5 and 25 μg/mL. The cells were then irradiated with NIR light using a Hydrosun-750 lamp for 1 hour. Cell viability, nanoparticle sorption / uptake and the rates of early and late apoptosis / necrosis were assessed at 1 and 24 hours post-irradiation.

MCF-7 cells exhibited the capacity for sorption of PEGylated graphene oxide nanoparticles, including under conditions of NIR irradiation. After 24 hours of incubation, the proportion of PC7+ (GO-positive) cells reached approximately 10 % at a concentration of 5 μg/mL and 50 % at 25 μg/mL. NIR-irradiated nanoparticles did not produce statistically significant changes in cell viability or apoptosis/necrosis rates. However, a trend towards increased cell death and apoptosis was observed 24 hours post-irradiation.

Keywords: graphene oxide nanoparticles, MCF-7, NIR irradiation, hyperthermia, apoptosis

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Оригинальная исследовательская статья

ОЦЕНКА ВЛИЯНИЯ НАНОЧАСТИЦ ОКСИДА ГРАФЕНА НА КЛЕТКИ ЛИНИИ MCF-7 ПОД ДЕЙСТВИЕМ БИК-ОБЛУЧЕНИЯ

М. Д. Долгих[1, 2], М. С. Бочкова[1, 2], В. П. Тимганова[2], С. А. Заморина[1, 2]*

[1] Пермский государственный национальный исследовательский университет, 614068, Россия, Пермь
[2] Институт экологии и генетики микроорганизмов, Уральское отделение Российской академии наук, 620049, Россия, Пермь

* Автор-корреспондент: mantissa7@mail.ru

Резюме: Поиск новых способов индукции апоптоза опухолевых клеток является актуальной задачей биомедицины. Учитывая уникальную теплопроводность графена, мы использовали данный наноматериал для нагрева клеток под воздействием ближнего инфракрасного излучения (БИК) в условиях *in vitro*. В работе применялись наночастиц оксида графена, покрытые линейным (лП-ОГм, Ø184 ± 73 нм) и 8-разветвленным (рП-ОГб, Ø1376 48 мкм) полиэтиленгликолем. Объектом исследования являлась клеточная линия аденокарциномы молочной железы MCF-7. Клетки культивировали 24 часа для образования монослоя. После этого добавляли наночастицы лП-ОГм и рП-ОГб в конечной концентрации 5 и 25 мкг/мл и затем облучали БИК-лучами (лампа Hydrosun 750) в течение 1 часа. Оценивали жизнеспособность клеток, поглощение/адгезию наночастиц клетками, а также параметры раннего и позднего апоптоза / некроза через 1 час и 24 часа после облучения.
Установлено, что клетки MCF-7 способны сорбировать наночастицы оксида графена, в том числе под действием БИК-облучения. Через 24 часа инкубации уровень PC7 + - клеток составлял 10 % (5 мкг/мл) и 50 % (25 мкг/мл). Показано, что наночастицы под воздействием БИК-облучения не оказывают статистически значимого влияния на жизнеспособность и показатели раннего и позднего апоптоза/некроза клеток линии MCF-7. Однако через 24 часа после облучения наблюдается тенденция к увеличению этих показателей.

Ключевые слова: наночастицы оксида графена, MCF-7, БИК-облучение, гипертермия, апоптоз

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Introduction

According to the World Health Organisation, breast cancer ranks first among all cancers in terms of new incidence. In 2020, it affected over 2,3 million people globally, with 685.000 recorded deaths [1]. One of the most prevalent forms of breast cancer is the luminal A subtype, which is most commonly diagnosed in older women [2]. The MCF-7 cell line — an epithelial tumour line representative of this subtype — is employed as a model system in the present study. MCF-7 cells serve as a suitable model for investigating various parameters, including cytotoxicity and functional, proliferative and secretory activities during interactions with different types of nanoparticles [3].

In recent years, devices emitting light in the near-infrared (NIR) range have gained increasing popularity. Typically represented by high-intensity lasers or specialised lamps, they are used across various fields of medicine, including the treatment of osteoarthritis [4]. Their therapeutic effect is attributed to the deep penetration of infrared radiation into bodily tissues and its action at the cellular level.

Interest in the application of NIR radiation in oncology is driven, in part, by the unique properties of graphene oxide (GO). Owing to its high thermal conductivity, GO is highly efficient at absorbing infrared light and converting it into thermal energy [5]. However, its direct use is constrained by inherent toxicity. To enhance biocompatibility and minimise toxic effects, GO was modified with linear and branched polyethylene glycol (PEG). The low cytotoxicity of such PEGylated nanoparticles has previously been demonstrated in T-lymphocytes (up to 25 μg/mL) [6], macrophages (up to 31,25 μg/mL) [7] and human peripheral blood mononuclear cells (up to 20 μg/mL) [8]. Thus, the minimal cytotoxicity of graphene oxide nanoparticles towards healthy tissues, combined with the accessibility of breast cancer sites to NIR therapy, renders this approach a promising strategy for tumour treatment.

The aim of this study was to investigate the effect of graphene oxide nanoparticles under NIR irradiation on MCF-7 cells. Specifically, the impact of GO nanoparticles on cell viability and apoptosis was assessed following NIR exposure in the presence of the nanoparticles.

Materials and Methods

Materials

Cell culture. MCF-7 cell line (PrimeBioMed, Russia).

Reagents. DMEM cell culture medium without phenol red (BioInn Labs, Russia); FBS (Capricorn Scientific, Germany), antibiotic-antimycotic solution (Himedia, India), L-glutamine (Biological Industries, Israel); Accutase (Capricorn Scientific, Germany); DPBS without Ca^{2+} and Mg^{2+} (PanEco, Russia); Zombie Aqua (BioLegend, USA), FITC Annexin V (BioLegend, USA); BSA (Life technologies Corporation, USA), NaN_3 (Dia-M, Russia).

Equipment. Hydrosun 750 NIR lamp (Hydrosun Medizintechnik, Germany, Table 1); CytoFLEX S flow cytometer (Beckman Coulter; USA); PST-60 HL shaker-thermostat (BioSan, Latvia).

Table 1

Hydrosun-750 lamp specifications

Nominal voltage 50/60 Hz	Power consumption	Infrared source USHIO	Average lifetime of the source	Radiation intensity (distance 32 cm = distance rod)	
				BTE31 color filter	No color filter
230 V AC	775 W	HPL 750/230X +	1500 hours	200 mW/cm² ± 10 %	225 mW/cm² ± 10 %

Graphene oxide nanoparticles. Graphene oxide (Ossila Ltd, UK) was coated with linear polyethylene glycol (LP-GOs) and 8-branched polyethylene glycol (BP-GOb). Nanoparticles were applied to cells at concentrations of 5 and 25 μg/mL. The characteristics of the nanoparticles are summarised in Table 2, with more detailed information available in a previously published study by our colleagues [9].

Table 2

Characteristics of graphene oxide nanoparticles coated with linear and 8-branched polyethylene glycol

Particle name	Size	Zeta potential
LP-GOs	∅184 ± 73 nm	− 31.70 ± 1.70, mV
BP-GOb	∅1376 ± 48 μm	− 53.56 ± 1.23, mV

Cultivation of MCF-7 cells. MCF-7 cells were cultured in DMEM growth medium supplemented with 10 % FBS, 2 mM L-glutamine and 1 % antibiotic-antimycotic solution. Re-seeding was carried out when the cells reached 80—90 % confluency. Cell cultures were maintained in a CO_2 incubator at 37 °C with 5 % CO_2. For the experiments, 125 μL of cell culture medium containing 45,000 cells was added to each well of a 96-well plate. Following the addition of the nanoparticles, the total volume per well was 150 μL. The overall experimental design is illustrated in Figure 1.

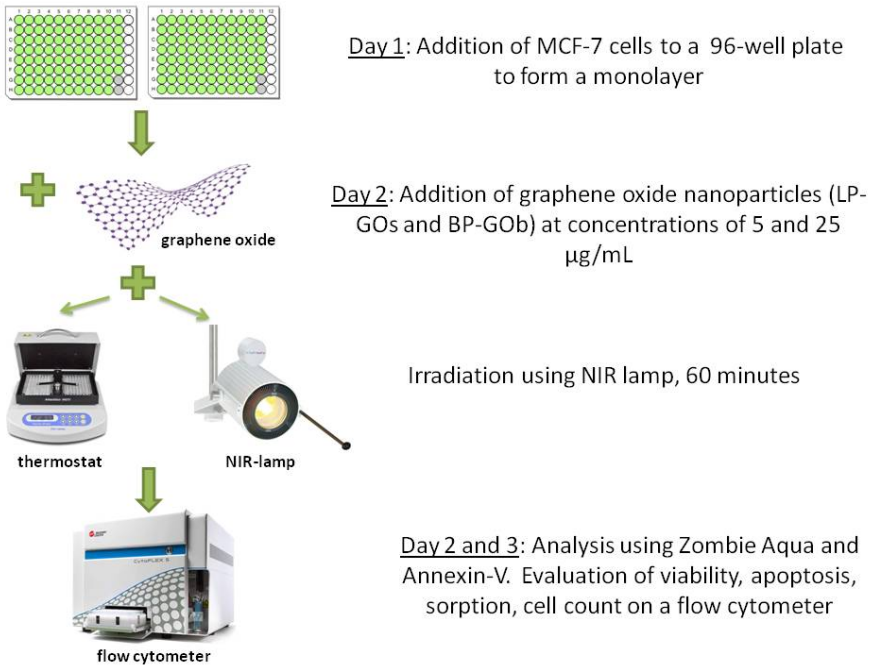

Fig. 1. The experimental design for investigating the effect of graphene oxide nanoparticles on MCF-7 cells under NIR irradiation

NIR irradiation. After 24 hours of cultivation of MCF-7 cells with PEGylated graphene oxide, two identical 96-well plates containing the cells were placed on a shaker thermostat until a temperature of 37°C was reached. One plate was then exposed to NIR irradiation for 1 hour, while the other was kept in the shaker thermostat for the same duration as a negative control. The effects were assessed 1 hour and 24 hours post-treatment.

Detachment of cells from the cell plate surface. Culture medium was transferred from the wells into test tubes. After resuspension, the wells were washed with DPBS. Accutase solution was then added to the wells, and the plates were placed on a thermo shaker at 340 rpm and 37°C for 10 minutes. Detached cells were collected into the test tubes. Cold DPBS was added to each well; the remaining cells were resuspended and transferred to the same tubes.

Sorption of NPs. A flow cytometry method detecting graphene oxide autofluorescence in the red spectral channel (PC-7) was employed to assess this interaction. Since this method cannot distinguish between nanoparticle adhesion to the cell surface and internalisation, the term «sorption» is used to describe the interaction between MCF-7 cells and the nanoparticles. A group not treated with GO served as the negative control.

Cell count determination. After detaching the cells from the culture plate surface, the volume of liquid in each tube was measured using a pipette. The samples were then diluted 40- and 70-fold. The absolute cell number was calculated by multiplying the event count per millilitre by the sample volume.

Assessment of viability and apoptosis. Zombie Aqua (ZA) dye was used to assess cell viability, while Annexin V was employed to detect apoptosis. DPBS was added to the tubes, and the cells were centrifuged for 10 minutes at 350g. Supernatants were removed, after which Zombie Aqua was added to the stained tubes and DPBS to the unstained ones. The tubes were incubated in the dark at room temperature for 30 minutes. Cold staining buffer (DPBS with 0.5 % BSA and 0.1 % NaN_3) was then added, and the cells were centrifuged at 4 °C for 10 minutes at 350g. The cells were resuspended in Annexin V solution and incubated in the dark at room temperature for 15 minutes. Annexin V-binding buffer was subsequently added, and the cells were kept on ice. The percentage of viable cells and apoptosis level were evaluated for singlet cells using gating (FSC-A vs. FSC-H).

Statistical analysis. Statistical processing was performed using GraphPad Prism 8 software with a parametric one-way ANOVA test and correction for multiple comparisons (Tukey's test). Four independent experiments were conducted. Significant differences compared to the control are indicated separately for the «thermostat» and «NIR-lamp» groups.

Results and discussion

Nanoparticle sorption. The study examined the ability of PEGylated graphene oxide to interact with MCF-7 cells. The results presented in Figure 2 show a clear positive correlation between the degree of GO nanoparticle sorption and their concentration in the medium. For instance, at a concentration of 5 μg/mL, the proportion of PC7 + cells reaches approximately 10 %, regardless of nanoparticle type, whereas at 25 μg/mL, the percentage exceeds 30 %.

Additionally, an increase in the sorption of LP-GOs by the cells 24 hours after treatment was observed, suggesting that the interaction is time-dependent and requires a certain period to reach saturation.

A review article on the uptake of GO by mammalian cells notes that nanoparticles ranging from 500 to 4000 nm are not internalised by MCF-7 cells but in-

stead adhere to their surface [10]. However, the authors emphasise that this effect is size-dependent. Therefore, it is likely that the BP-GOb nanoparticles (1—5 μm) used in this study primarily adhere to the cell surface, whereas LP-GOs are more likely to penetrate the cells.

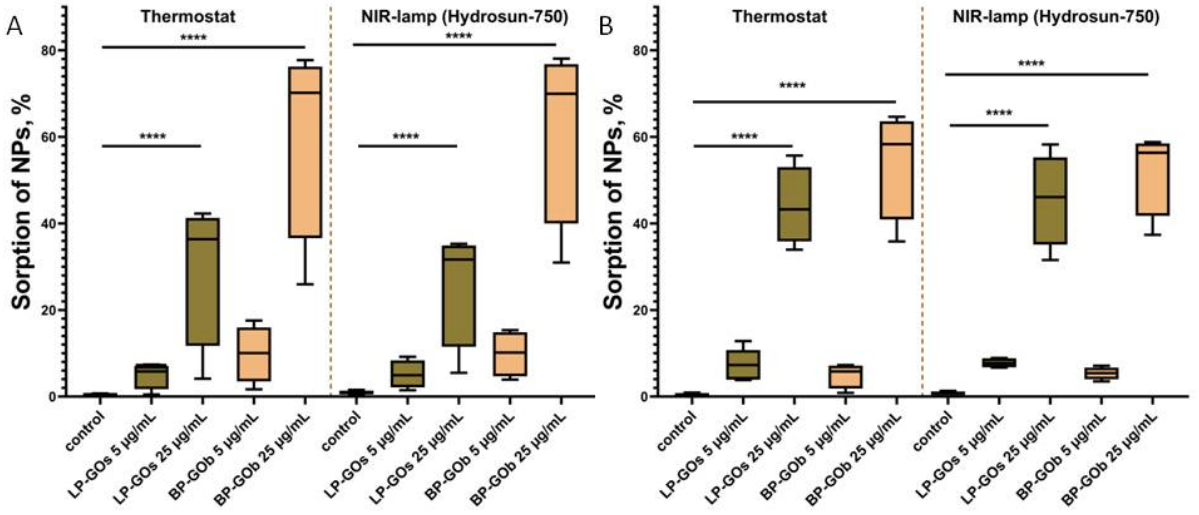

Fig. 2. Sorption of PEGylated graphene oxide by MCF-7 cells
1 hour after irradiation (A)
and 24 hours after irradiation (B).
N = 4. Median and interquartile ranges (IQR) are presented. **** – $p \leq 0.0001$

Cell counting. Cell counting was performed immediately after detachment from the culture plate surface. As shown in Figure 3, there were no statistically significant differences in the number of MCF-7 cells between the control group and the groups treated with PEGylated GO or exposed to NIR irradiation. Thus, PEGylated graphene oxide nanoparticles and NIR irradiation had no measurable effect on MCF-7 cell numbers.

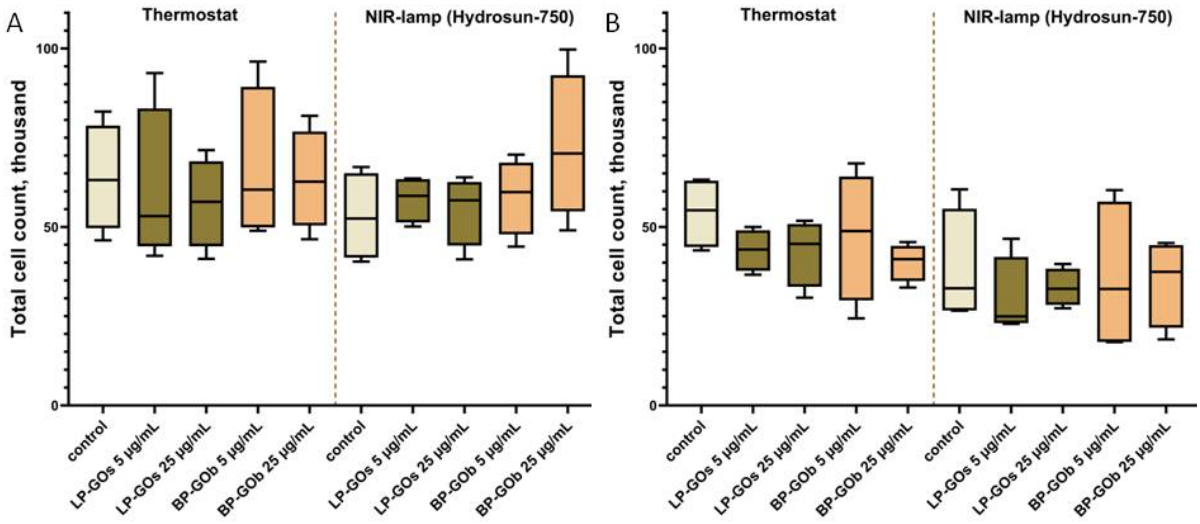

Fig. 3. MCF-7 cell counts 1 hour after irradiation (A)
and 24 hours after irradiation (B). N = 4.
Median and IQR are presented

Viability. Cell viability was assessed using Zombie Aqua dye, which penetrates cells with damaged membranes. The study showed that PEGylated graphene oxide and NIR irradiation had no statistically significant effect on MCF-7 cell viability (Fig. 4). However, a tendency towards decreased viability was observed 24 hours after irradiation.

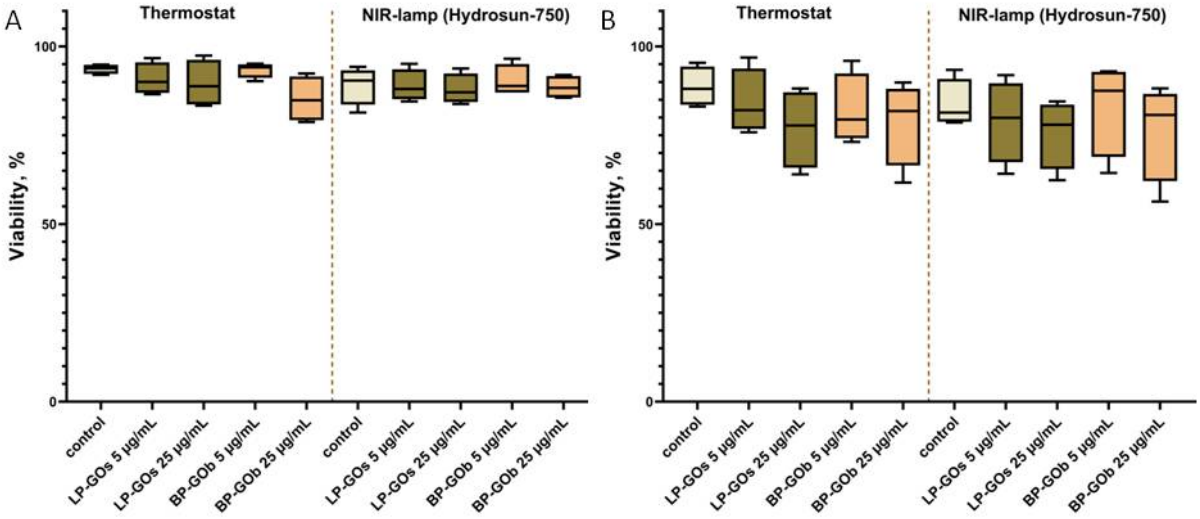

Fig. 4. MCF-7 cell viability 1 hour after irradiation (A) and 24 hours after irradiation (B). N = 4, median and IQR are presented

Apoptosis. The experimental results showed that PEGylated GO and NIR irradiation had no significant effect on early apoptosis (ZA^- Annexin V^+) or late apoptosis / necrosis (ZA^+ Annexin V^+) in MCF-7 cells (Fig. 5). However, 24 hours after irradiation, a trend towards an increase in these indicators was observed. In similar studies, graphene oxide coated with 8-hydroxyquinoline induced apoptosis in MCF-7 cells at a concentration of 0,5 mg/mL after 48 hours of cultivation [11]. This suggests that the apoptotic response in the present study may reflect a delayed effect.

Since 2010, graphene-induced hyperthermia of tumour cells has been studied both *in vitro* and *in vivo*. Experiments in mice have shown that PEGylated graphene oxide nanoparticles exhibit highly efficient passive accumulation in transplanted tumours, with relatively low retention in the reticuloendothelial system. NIR irradiation using an 808 nm diode laser resulted in highly effective tumour ablation following intravenous nanoparticle injection. Moreover, no apparent side effects were observed in the treated mice, as confirmed by histological, haematological and biochemical analyses [12].

Over the past 15 years, notable progress has been made in the study of graphene as an agent for local tumour hyperthermia [13]. In 2023, ultra-small graphene oxide nanoparticles (average size 30 nm) modified with polydopamine were synthesised. These nanoparticles demonstrated good biocompatibility, excellent photothermal performance and a high drug-loading capacity for doxorubicin [14]. Overall, conjugated nanoparticles have been regarded as a promising approach to combine chemotherapy and phototherapy in tumour treatment.

Yet no clinical studies have been performed. In addition, the cytotoxic mechanisms of nanoparticles against tumour cells remain poorly understood. Besides apoptosis induced by the temperature rise during hyperthermia [15], increased intracellular

production of reactive oxygen species (ROS) following nanoparticle treatment has been reported [16]. Elevated ROS levels cause damage to proteins, nucleic acids, lipids, membranes and organelles, ultimately leading to cell death.

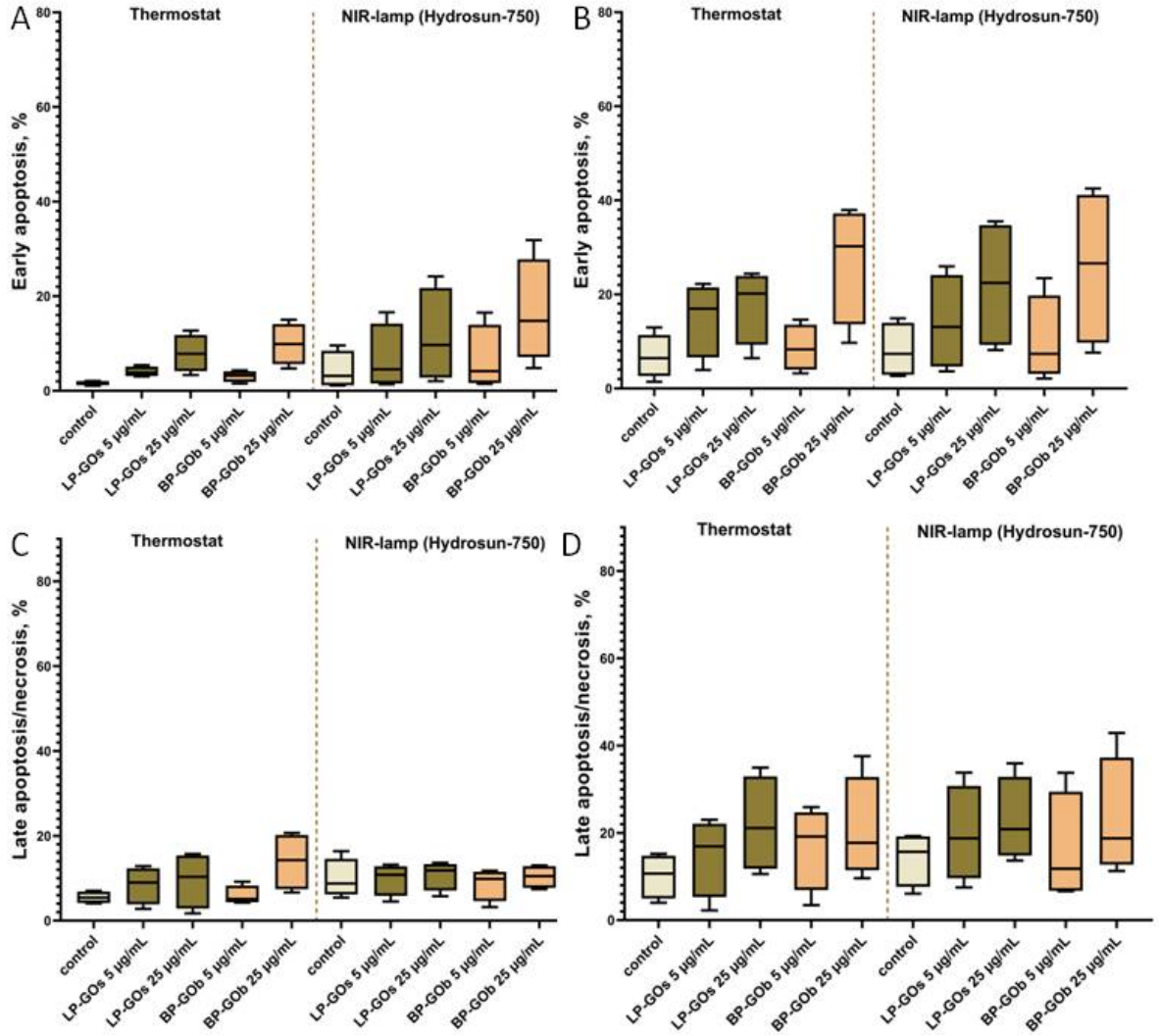

Fig. 5. Early apoptosis of MCF-7 cells 1 hour
after irradiation (A) and 24 hours after irradiation (B).
Late apoptosis/necrosis of MCF-7 cells
1 hour after irradiation (C) and 24 hours after irradiation (D).
N = 4, median and interquartile ranges (IQR) are presented

Thus, our study demonstrated that PEGylated graphene oxide, irrespective of the type of modification (linear or branched PEG) and the exposure method (thermostat storage or NIR irradiation), had no effect on cell count, viability or apoptosis. Consequently, these exposure conditions are not toxic to MCF-7 tumour cells (Fig. 6).

PEG-coated graphene oxide can be sorbed by MCF-7 cells, indicating its potential for further investigation. Future experiments will focus on optimising the extracellular environment to induce apoptosis following nanoparticle application. Overall, it is crucial to develop a technique that selectively eliminates cancer cells while preserving healthy tissue. Achieving this requires careful optimisation of parameters such as nanoparticle concentration, exposure time and other factors influencing the efficacy and safety of the treatment.

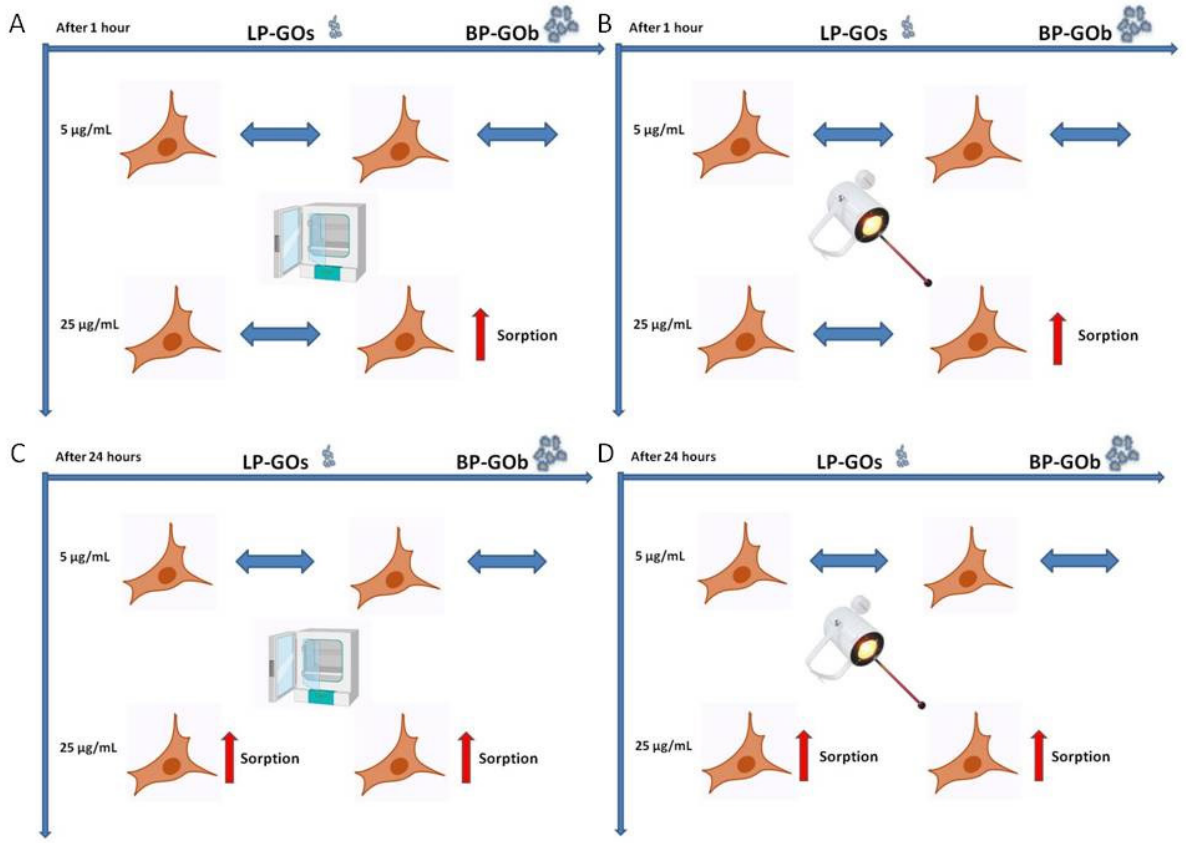

Fig. 6. Summary of the findings. Effects were evaluated 1 hour (A and B) and 24 hours (C and D) after irradiation

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The authors

Maria D. Dolgikh, laboratory assistant, bachelor, laboratory of Cellular Immunology and Nanobiotechnology "IEGM of the Ural Branch of the RAS", Perm, Russia.

ORCID: 0009-0002-1418-4157

Maria S. Bochkova, PhD, researcher, laboratory of Cellular Immunology and Nanobiotechnology "IEGM of the Ural Branch of the RAS", Perm, Russia.

ORCID: 0000-0001-5784-6224

Valeria P. Timganova, PhD, researcher, laboratory of Cellular Immunology and Nanobiotechnology "IEGM of the Ural Branch of the RAS", Perm, Russia.

ORCID: 0000-0003-4581-1969

Svetlana A. Zamorina, PhD, D.Sci.Biol., leading researcher, laboratory of Cellular Immunology and Nanobiotechnology "IEGM of the Ural Branch of the RAS", Perm, Russia.

ORCID: 0000-0002-6474-1487

Об авторах

Мария Денисовна Долгих, инженер, бакалавр, лаборатория клеточной иммунологии и нанобиотехнологии, Институт экологии и генетики микроорганизмов, Уральское отделение Российской академии наук, Пермь, Россия.

ORCID: 0009-0002-1418-4157

Мария Станиславовна Бочкова, кандидат биологических наук, научный сотрудник, лаборатория клеточной иммунологии и нанобиотехнологии, Институт экологии и генетики микроорганизмов, Уральское отделение Российской академии наук, Пермь, Россия.

ORCID: 0000-0001-5784-6224

Валерия Павловна Тимганова, кандидат биологических наук, научный сотрудник, лаборатория клеточной иммунологии и нанобиотехнологии, Институт экологии и генетики микроорганизмов, Уральское отделение Российской академии наук, Пермь, Россия.

ORCID: 0000-0003-4581-1969

Светлана Анатольевна Заморина, доктор биологических наук, ведущий научный сотрудник, лаборатория клеточной иммунологии и нанобиотехнологии, Институт экологии и генетики микроорганизмов, Уральское отделение Российской академии наук, Пермь, Россия.

ORCID: 0000-0002-6474-1487