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The effect of gold nanoparticles and irradiation on healthy and tumor human lung cells

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► ***Original article***

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ABSTRACT

Background: Cancer cells may develop resistance to radiation which results in decreasing effects of radiation therapy. The effectiveness of high dose radiation can be increased with previously delivered low dose. Also, the approach that has been used to enhance the efficacy of radiation is to increase the radiosensitization. The focus of interest is on usage of gold-based nanoparticles as radiosensitizers. Modification of gold nanoparticle surface may increase its effectiveness in cells. The aim of the study was to prepare and characterize nanogold/β-cyclodextrin formulation (nAu/β-CD) and to investigate cytotoxicity and differences in radio-adaptive response of irradiated normal and malignant cell lines. ***Materials and Methods***: Nanoparticles distribution and zeta-potential of nAu/β-CD were obtained by dynamic light scattering (DLS). Cell lines MRC-5 and A549 were pretreated with 0.05 μM nAu/β-CD formulation and irradiated in single and double regimes. The MTT test was performed after 24 h and 72 h of recovery. ***Results:*** Nanogold particles didn't express cytotoxic effect on MRC-5 and A549 cells. Tested nanoformulations with nAu/β-CD ratio 1:1 achieved the best cytotoxic effect against A549 cell line. Pre-treatment with low dose (0.05 Gy) prior to therapeutic dose (2 Gy) and application of nAu/β-CD nanoformulation decreased the cell survival in all investigated samples, showing diverse effects on normal and tumor cells. ***Conclusion:*** Results indicate potential selectivity and increased efficiency of both applied radiation and pre-treatment with nAu/β-CD regarding the malignant cells, while sparing the normal tissue from radiation damage, which could be beneficial for the radiotherapy.

Keywords: *gold nanoparticles, β–cyclodextrin, cytotoxicity, human cell lines, radioadaptive irradiation.*

INTRODUCTION

Radiation therapy, along with chemotherapy and surgery is the major therapeutic strategies for cancer treatment. It implies accurate delivery of high intensity ionizing rays to the tumor tissue, resulting in death of the tumor cells. Its disadvantage is the potential injury of the tumor -surrounding healthy tissue. Moreover, the cancer cells can develop resistance to the radiation. The occurrence of this resistance may result in reduced efficiency of radiation therapy. The efficacy of high dose radiation can be increased with previously delivered low dose irradiation (LDIR). This is a well recognized phenomenon named dose radiation induced adaptive response. Upon LDIR treatment, specific cellular mechanisms are activated and thus avoiding cells resistance or adaptability to further high dose [(1)]. The phenomenon of

radioadaptive response consists of the radioadaptive response in the narrow sense, then hyper-radiosensitivity and induced radioresistance.

In order to strengthen the response of tumor tissue to radiotherapy, along with the spearing of healthy cells and tissues, many researches were conducted aiming to obtain a radioadaptive response in terms of saving healthy tissue and, in contrast, causing hyper-radiosensitivity in tumor cells. In both phenomena, low radiation doses that are delivered prior to high doses of radiation have an important role. Low doses are named priming doses and latter high doses are named challenging doses [2,3]. Hyper-radiosensitivity is not completely described, but it was shown that it is associated with p53 induced apoptosis [3,4]. Also, it is considered that priming doses are not sufficient to activate DNA repair mechanisms [5].

Furthermore, one of the approaches that has been used to enhance radiation efficiency while reducing its harmful effects is increasing the radiosensitization of tumor tissue [6]. The radiation sensitization is a process of increasing the susceptibility of tumors to injuries caused by radiation exposure. Radiosensitizers are agents that increase the effects of radiation therapy. There is a focus of interest on the usage of metal (mainly gold) based nanoparticles as radiosensitizers [7]. The use of nanomaterial radiosensitizers is also called Nanoparticle Enhanced X-ray Therapy (NEXT) [8].

Densely packed metal particles can selectively scatter and/or absorb high-energy radiation. This permits efficiently targeting of components within the cancer cells conceding for more intended and consolidated damage [9,10]. This effect increases the strength of main radiation events and consequently results in decrease of the therapeutic radiation dose and further mitigating the damage to the healthy tissue. Also, the underlying interaction of nanoparticles with physiological fluids is important for understanding their biological impact, and can perhaps be exploited to avoid harmful effects [7,11]. It is known that gold nanoparticles (nAu) are not inert in biological systems, causing reactive oxygen species production, cytotoxicity, cytokinesis arrest and apoptosis [12]. Further, biocompatible, high Z gold nanoparticles are an ideal material for photosensitization. They are well absorbed into systemic circulation and have good permeation into the tumor tissue. Also, they can vary in size and shape and have the ability to bind targeted moieties such as antibodies so can be easily conveyed and delivered [11,13]. Dose levels of golden nanoparticles can be optimized due to their characteristic to be easy for imaging and quantifying [14].

Modification of gold nanoparticle surface by attaching bioactive ligands (such as drugs or proteins) improves the possibility for their entrance into the cells and increases its effectiveness. So formed gold complexes have recently gained considerable attention as a class of compounds with different pharmacodynamic and pharmacokinetic properties with strong cell growth–inhibiting effects [15,16]. The investigations about use of coated gold nanoparticles in medicine are in rise especially in advancement of therapeutic strategy for cancer treatment [17].

Cyclodextrins (CDs) are considered biocompatible at low concentrations and they have been employed in biomedical systems [18]. These soluble, nontoxic molecules become a good choice as they can form both channel and cage structures incorporating nanosize metal guests with its cyclic oligosaccharide containing internal cavities. They are made up of six, seven, or eight glucose units connected in a large ring, called α-, β-, or γ-CD, respectively. Among the three CDs, β-CD is the most widely used because its internal cavity diameter ranges from 0.6 to 0.65 nm [19].

It is well known that different types of endocytosis serve to cellular uptake of gold nanoparticles. The efficacy of endocytosis depends on cell type, the mechanism of interactions with the cell medium and inner fluids, as well as on size, shape, coating of applied nanoparticles and ability to form aggregates [11,13].

The cytotoxicity and sensitization investigations that use coated gold nanoparticles alone or in combination with different

irradiation models are actual and in function of the advancement of therapeutic strategy for cancer treatment. The *in vitro* experiments are simple and easily repeatable way to lay the foundation for future *in vivo* research and potential involvement in clinical practice.

The aim of our study was to prepare and characterize optimal nAu/β-CD formulation as well as to monitor its effects and differences in radioadaptive response of irradiated both healthy and malignant cell line.

MATERIALS AND METHODS

Preparing of nanoformulation

Nanoformulation was prepared according the following procedure: First, the β-cyclodextrin (Sigma Aldrich, USA) stock solution with concentration of 1 mg/mL (880 μmol/L) was prepared with demineralized water (17.5 MΩ) and sonicated (power rating 700 W; frequency 20 kHz) for 10 min at 22° C. The concentration of nano Au solution (Kolloidales Gold, Silberstab, dispersion 30 ppm, Fa Matschewsky, Weimar, Germany) was 152 μmol/L.

The solutions of two nanoformulations nAu/β -CD 1:1 and 1:2 were prepared by mixing stock solutions of nano Au (50 μmol/L) with two different β-cyclodextrin solutions in demineralized water (50 and 100 μmol/L respectively). The resulting solutions were sonicated for 10 minutes at 22° C.

Results of distribution (the mean hydrodynamic diameter) and zeta-potential of nanoformulation (nAu/β-CD) 1:1 and 1:2 were obtained by dynamic light scattering (DLS) using Zetasizer Nano ZS, Malvern. All DLS measurements were done in triplicate, at wavelength 633 nm with a detection angle of 173°, at 22° C.

Cell preparation and treatment with nAu/β-CD

Experimental procedure was performed using two cell lines, human lung fibroblasts MRC -5 (ATCC CCL 171) and human lung adenocarcinomic alveolar basal epithelial cells A549 (ATCC CCL 185). Cell lines were cultured in Dulbecco's modified Eagle's medium with 4.5 g/L glucose (DMEM, Gibco BRL, UK), 10% FBS (fetal bovine serum, Sigma) and containing antibiotic/antimycotic solution (Sigma). The cell lines were grown in incubator at 37° C with 100% humidity and 5% CO_2 (Heraeus). The cells were passaged twice a week; those used in the experiments were in the logarithmic phase of growth, between the third and the tenth passage. The number of cells and their viability were determined using dye exclusion test (DET) with 0.1% trypan blue (data not shown) [20].

The samples were treated with nAu alone, and nano Au/β-cyclodextrine formulations (nAu/β-CD 1:1 and 1:2) in a concentration range from 0.05 to 50nmol/L. After 24 h incubation with nanoformulations, the MTT test was performed.

MTT assay

Cytotoxicity from the aspect of cell survival was evaluated by tetrazolium colorimetric MTT assay (Sigma) according to Mosmann [21]. Cytotoxicity was expressed in percentages according to the formula:

$$CI = (1 - A_s / A_k) \times 100\%$$

where A_k was the average absorbance of the control samples and A_s was the average absorbance of the samples containing the examined substance. All samples were analyzed in quadruplicates.

Irradiation scheme and procedure

Viable cells were seeded in flasks (25 cm^2, Sarstedt) at concentration of 1×10^6 cells / 10 mL. Flasks were incubated 24 h at 37° C, with 100% humidity and 5% CO_2. The IC50 concentration of nAu/β-CD (1:1) nanoformulation was added to flasks 30 minutes before irradiation.

Flasks with control and treated cells were added to Plexiglas phantom for irradiation. The CT scans of phantom were made (Somatom 4, Siemens) and imported to irradiation planning system Electa XIO, version 4.62. The plan with

two fields and isodose-distribution in a range from 95 – 107% was done through the planning software. Distribution of ionizing radiation was performed through linear accelerator Varian 600DBX, with 6 MV energy and dose rates of 80 MU/min for doses of 0.05 and 0.2 Gy, and 400 MU/min for dose of 2 Gy (as described in Djan *et al.* 6458 and 6459) [3,22]. The samples of both cell lines were irradiated with 0.05, 0.2 and 2 Gy.

Four hours later, selected samples were additionally irradiated to achieve the regime 0.05 Gy + 0.2 Gy, 0.05 Gy + 2 Gy and 0.2 Gy + 2 Gy.

Post-irradiation treatment of cells

Twenty-four hours after irradiation, cells were tripsinized and re-suspended in fresh medium. Cell viability and the total cell number was measured (DET test, data not shown). Both fibroblasts and lung carcinoma cells were plated at 5×10^5 cells/well in 96-well microplates (Falcon) and were taken in incubator for 24 or 72 h recovery time. The MTT test was performed after the recovery time in a fresh medium.

Data analysis

In this study, all the experiments were repeated. The *in vitro* cytotoxicity testing was performed in quadruplicate for each concentration point. Calculation of mean values, standard deviations (SD), coefficients of variation (CV) and all statistical processing were done in Microsoft Office Excel program. The IC50 value of tested substances was determined by median effect analysis, and it is defined as the dose of substance that inhibits cell growth for 50% in comparison to control sample.

RESULTS

Complexes in the obtained nanoformulation have diameter from 5 to 16 nm, where the most of the particles have size of about 8 nm (28.6%) (figure 1A, green line). The particle size distribution by number of colloidal gold nanoparticles at concentration of 50 μmol/L showed that most abundant were particles with diameter of approximately 5 nm (26.6%), and the others were 2.5 – 10 nm in diameter (figure 1A, red line). The mean zeta potentials of used nano Au/β-cyclodextrine (nAu/β-CD 1:1 and 1:2) formulations were -6.8 and -3.9 mV respectively (figure 1B). Zeta potential represents the surface charge of nanoparticles as the degree of electrostatic repulsion in an investigated medium. It is a very important parameter which complements the results of DLS measurements giving an insight into specific characteristics of nanosystem. Generaly, zeta potential values may indicate the possible interactions with biological model the nanosystem will be applied in. Besides, the changes in zeta potential may give an insight into electrostatic interactions between components within a nanosystem. Obtained values of ζ-potential indicate the mean charge of applied nanoformulation which can be considered as stable [23].

In the first part of the experiment we investigated the cytotoxic potential of nanogold alone and nanoformulations nAu/β-CD 1: 1 and 1:2. Cultures of MRC-5 and A549 cells were seeded in 96-well plates, with a selected formulation in concentration range from 0.05 to 50 nmol/L. The MTT test was performed after 24 h of incubation. The results are shown in table 1.

In tested range of concentrations, none of the applied formulations affected the MRC-5 cell line. Regarding the A549 cell line, the formulation of nAu/β-CD 1:1 was more effective and showed lower IC50 than nanoformulation nAu/β-CD 1:2. The nAu alone was inactive on both cell lines.

Based on the results of MTT test, the nAu/β-CD 1:1 nanoformulation was selected for further testing with radiation and the samples of both cell lines MRC-5 and A549 were treated with the IC50 concentration 30 minutes before irradiation.

Formulation with predominant 8 nm nAu/β-CD particles led to decreasing of survival percentage in all investigating samples. The survival of MRC-5 cells treated with nAu/β-CD was 18.9% lower in 24 h regime and lower for 24.81% in 72 h regime, compared to control. In A549 samples, the percentage of survival was significantly lower (for even 49%), i.e. 55.04% (figure 2).

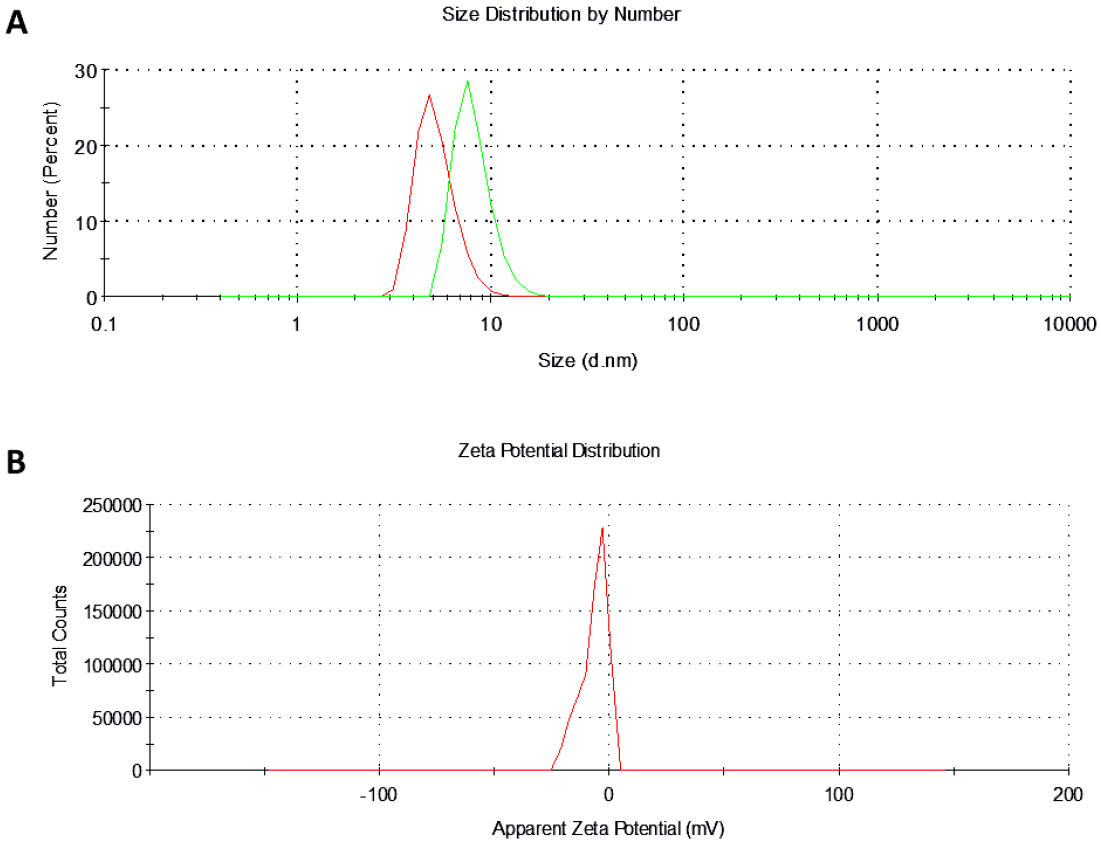

Figure 1. A) – DLS measurements of nano Au (red line) and nanoformulation nAu/β-CD (green line). **B)** – Mean zeta potential of nanoformulation nAu/β-CD.

Table 1. The IC50 value [nmol/L] for nAu, nAu/β-CD 1:1 and nAu/β-CD 1:2 after 24 h incubation in human fibroblasts MRC-5 and lung adenocarcinomic cells A549 obtaoned with MTT test.

		IC50 [nmol/L]	
Nanoparticles		MRC-5	A549
1	nAu	*	*
2	nAu/β-CD 1: 1	*	27
3	nAu/β-CD 1: 2	*	42

* The IC50 value was not achieved within the applied concentration range.

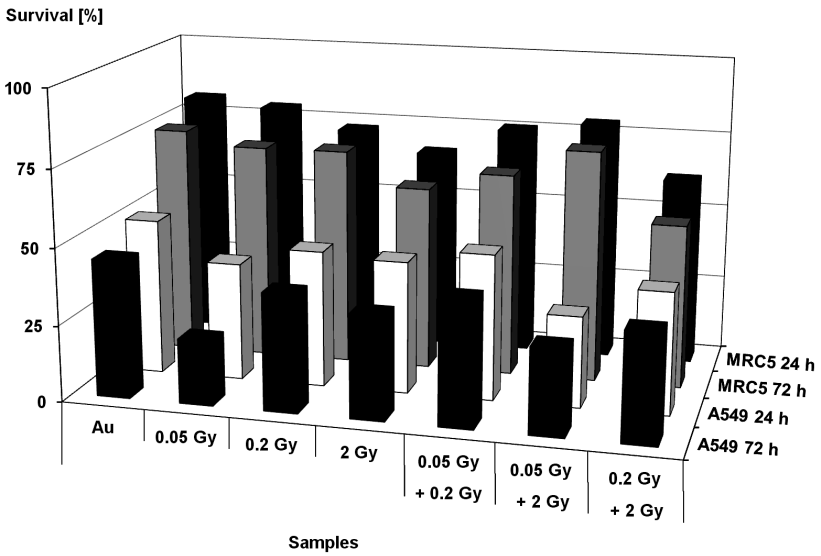

Figure 2. The survival trend [%] of irradiated MRC-5 and A549 cells pre-treated with nAu/β-CD 24 h and 72 h after recovery time.

Pair-wise comparisons of MRC-5 cells (figure 3 – A) irradiated with priming dose of 0.05 Gy and subsequently challenging dose of 0.2 Gy, with those cells irradiated only with challenging dose revealed differences between these two groups of samples. We note that in the first (pre-irradiated) group, the percentage of cytotoxicity was increased in both: with or without added nAu/β-CD particles in comparison to a group that is irradiated only with 0.2 Gy. So, we may conclude that radioadaptive response was not achieved at applied doses.

In samples irradiated with 0.2 Gy and then with 2 Gy, related to samples irradiated only with 2 Gy, there was decreased cytotoxicity in the group that is only irradiated, but also increased cytotoxicity in the group pre-treated with nAu/β-CD nanoparticles. Pre-irradiation with 0.05 Gy caused remarkable decrease of cytotoxicity compared to the group treated only with radiation dose of 2 Gy. Experimental results indicate that pre-irradiation with low dose (0.05 Gy) prior to the application of the usual therapeutic dose (2 Gy) protects normal fibroblasts from radiation damage.

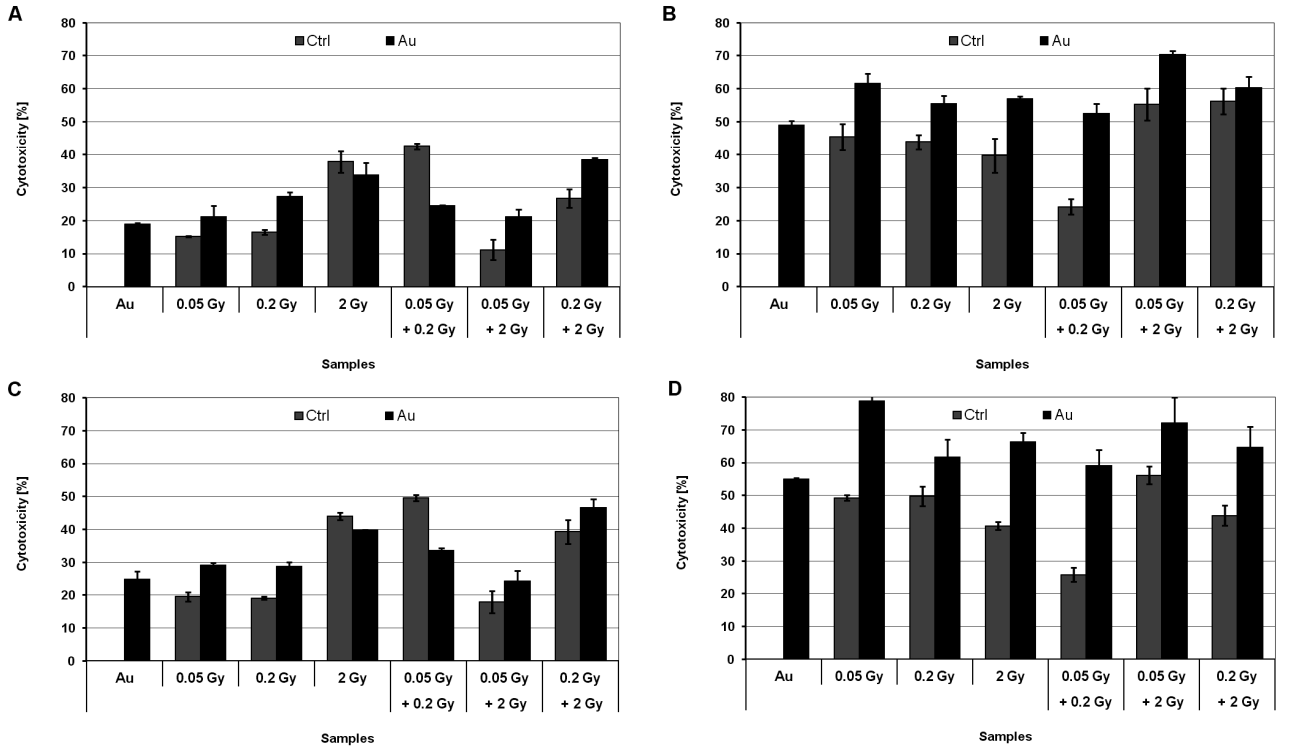

Figure 3. The cytotoxicity [%] measured in control (Ctrl) and samples treated with nAu/β-CD formulation (Au) of irradiated MRC-5 cells after 24 h recovery time **(A)**, A549 cells after 24 h recovery time **(B)**, MRC-5 cells after 72 h recovery time **(C)** and A549 cells after 72 h recovery time **(D)**.

If we compare pairs of A549 cell samples treated in 24 h recovery time regime (figure 3 – B), in which one group is irradiated with both lower and then a higher dose, and the other only with higher dose, we note the following: In samples with 0.05 + 0.2 Gy radiation applied, a percentage of cytotoxicity in both groups, the irradiated only and irradiated with added nanogold particles, is decreased in comparison to a group that is irradiated only with 0.2 Gy. Obtained results indicate the radioresistance phenomenon opposite to the MRC-5 cell line of normal fibroblasts. In the group of samples irradiated with 0.2, and then with 2 Gy, in relation to the group irradiated only with 2 Gy, there was an increase in percent of cytotoxicity in both irradiated groups. Also, pre-radiation with 0.05 Gy significantly influenced the

increase of cytotoxicity in this group (0.05 + 2 Gy) compared to the group treated with radiation dose of only 2 Gy. These experimental results indicate that pre-treatment with low doses prior to the application of the usual therapeutic dose (2 Gy) on the malignant cell line leads to an increase in the efficiency of both the applied radiation and the pre-treatment with gold nanoparticles.

In experimental groups both A549 and MRC-5 cells treated with nAu/β-CD and the selected radiation doses in the 72 h regime (figures 3 – C and D), an identical trend was observed in relation to groups irradiated in 24 h regime with increased percentage of cytotoxicity in all samples. It indicates that longer recovery time affects the decrease in survival in the samples of both cell lines. This experimental result may be attributed to the manifestation of cellular damage initiated during pre-treatment and expressed in the subsequent cell divisions.

In the A549 cell line, at all selected doses and radiation regimens, there was a significant increase in the percentage of cytotoxicity in both irradiated and nanogold–pretreated samples, in comparison to the same experimental conditions for the MRC-5 cell line. These results attribute to the selectivity of the applied treatment to malignant cell line.

DISCUSSION

Beta cyclodextrin has the ability to form inclusion complexes with other moieties in the solution. So, it can be assumed that b-cyclodextrin molecules form at first complexes which than form clusters with nano Au particles. Our results are in accordance with theoretically calculated diameter of b-cyclodextrin cavity which is approximately 0.7 nm, and the side rim depth is about 0.8 nm [24]. It is known that b-cyclodextrin can form complexes with different drug molecules by making an inclusion complex (drug moiety enters the b-cyclodextrin cavity) [25]. It is also assumed that once formed, these complexes are stabile and mutually independent. The literature data also indicate that cyclodextrins can form both – inclusion and non-inclusion complexes which can further form water-soluble aggregates and agglomerates [25, 26].

In present study, MRC-5 and A549 cells were pretreated with chosen nano Au / beta-cyclodextrin formulation and irradiated in a single and double regime. The cell growth was stopped, cells were re-seeded in 96-well microplates and citotoxicity test was performed after 24 h and 72 h of recovery. Pre-treatment with nanoformulation nAu/β-CD was toward investigation of cytotoxicity of used formulation alone, as well as its possible adaptive and/or sensitizing effect on cell lines treated with irradiation.

It is known that the particle size and, consequently, cellular uptake have direct impact on cytotoxicity. According to Pan *et al*. [27], the particles of approximately 2 nm play a pivotal role in inducing cytotoxicity in HeLa cells *in-vitro*.

Also, the observed decrease in survival rates, depending on the cell line and the duration of recovery, suggests the selective cytotoxicity of the nAu/β-CD formulation for malignant cells. It correlates with the investigation of Patra *et al*. [28] who noticed that A549 cell line shows high sensitivity to the treatment with nAu particles which induce concentration-depended cell death. Also, this group of authors has concluded that specificity of the induction of apoptotic response in A549 cells implies that nAu do not universally target all cell types [28].

The role of nanoparticle diameter in the eventual sensitization outcome depends on the balance between the impacts of size on uptake as well as size effect on photon emission. Therefore, increasing the uptake of particles with larger diameter into cells may have the most optimal outcome [6]. Niidome *et al*. [29] have shown that the toxic potential is triggered by the surface modification of the gold nanoparticles. Independent studies conducted by multiple groups of authors using polyethylene-glycol (PEG) coated gold nanoparticles showed increased radiotherapeutic efficacy of the formulation [30-32]. To our best knowledge, no published data are available about the effects of nanogold formulation with beta CD in

combination with irradiation *in vitro*. So, the results of experiments which show that irradiation decreases survival percentage in samples pretreated with nAu/β-CD were not published yet.

The radioadaptive response is well defined in literature as an induction of radioresistance to subsequent higher doses of radiation by priming irradiation with low radiation doses [33-36]. In this study, the presumption of the achieved radioadaptive response could be indirect, by monitoring which one of the irradiated samples had a decrease in percent of cytotoxicity using the MTT test.

It is notable that in both cell lines after priming dose of 0.05 Gy radioadaptive effect occurs after radioadaptive irradiation (priming dose followed by challenging dose). In MRC-5 normal fibroblasts cell line sparing effect is present, and in A542 tumor cell line hyper-radiosensitivity is present. This is in accordance with results from Ojima *et al*. [36] who investigated the *in-vitro* induction of radio-resistance in MRC-5 cells to subsequent higher doses of radiation by a priming irradiation with low doses. Also, Jiang [37] and Li [38] confirmed that low-dose ionizing radiation (LDIR) induces hormesis, exerts an adaptive effect on normal mammalian cells and stimulates cell proliferation; however, this effect is absent in cancer cells *in vitro*. Numerous investigations of radiosensitation phenomenon provided scattered explanations of underlying mechanisms, but this phenomenon, as well as radioadaptation after priming doses, are not fully understand [5,39-42]. Also, several studies have reported the sensitization effects of gold nanoparticles on cells irradiated with low energy radiation [43, 44]. Joh *et al*. [45] found that the PEG coated gold nanoparticles not only enhanced the radiation effects on glioblastoma tumor cells *in vitro* but also showed significantly higher brain endothelial cell death. The influence of gold nanoparticles to A549 cell line was shown in other studies [46, 47]. Studies by Bobyk *et al*. on glioma cell line and mice models of glioma showed a similar increase in efficacy and improved survival rate with naked gold nanoparticles in combination with radiation therapy [48].

CONCLUSION

Nanogold particles did not express cytotoxic effect on MRC-5 and A549 samples at applied range of concentrations after 24 h incubation time. Tested nAu/β-CD nanoformulations were active only against A549 cell line, and formulation with nAu/β-CD ratio 1:1 achieved better cytotoxic effect. Priming dose of 0.05 Gy applied prior to challenging (therapeutic) dose of 2 Gy showed selectivity and diverse effects on normal and tumor cells, beneficial for the radiotherapy treatment, regardless the application of nAu. The recorded effects comply sparing of the normal cells (MRC-5 cell line) and hyper-radiosensitivity in tumor cells (A549 cell line). Furthermore, application of nanogold particles made these effects more pronounced. The results of our research give hope that, after extensive *in vitro* and *in vivo* trials with nAu/β-CD formulation and irradiation, as well as insight into the molecular mechanisms that follow the phenomena we observed, such treatment could have a clinical significance.

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***Conflicts of interest*:** *Declared none.*

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