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Research Article

Efficient Induction of Apoptosis in Lung Cancer Cells Using Bismuth Sulfide Nanoparticles

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Background: The use of nanomaterial-based radiosensitizers to improve the therapeutic ratio has gained attraction in radiotherapy. Increased radiotoxicity applied to the tumor region may result in adverse impact on the unexposed normal cells to the radiation, a phenomenon known as radiation-induced bystander effect (RIBE).

Objectives: This study aimed to investigate the effect of Bi_2S_3 @BSA nanoparticles (NPs) as radiosensitizers on the enhancement of bystander response in non-irradiated cells.

Materials and Methods: Lung carcinoma epithelial cells were exposed to 6 MV x-ray photons at different doses of 2 and 8 Gy, with and without Bi_2S_3 @BSA NPs. The irradiated-cell's conditioned medium (ICCM) was collected and incubated with MCR-5 human fetal lung fibroblasts.

Results: This study showed that ICCM collected from 2-Gy-irradiated A549 cells in the presence of Bi_2S_3 @BSA NPs reduced the cell viability of MCR-5 bystander cells more than ICCM collected from irradiated cells without NPs (P<0.05), whereas such a difference was not observed after 8-Gy radiation. The mRNA expression of the BAX and XPA genes, as well as the cell death rate in MCR-5 bystander cells, revealed that the Bi_2S_3 @BSA NPs significantly improved bystander response at 2-Gy (P<0.05), but the efficacy was not statistically significant after 8-Gy Irradiation.

Conclusion: The results indicated that the presence of NPs did not affect bystander response enhancement at higher concentrations. These findings highlighted the potential use of radiation-enhancing agents and their benefits in radiotherapy techniques with high doses per fraction.

Keywords: Bismuth Nanoparticles; Bystander Effect; Lung cancer; Radiation Therapy

1. Background

Targeted therapy based on radiation has become an ideal approach in cancer research (1). Radiation therapy is divided into external and internal categories based on the location of the radiation source and applied in different cancers (2, 3). Although internal radiation

therapy delivers a higher dose of radiation with fewer side effects, it is used less frequently due to the need for invasive methods to place the radiation source in the proximity of the tumor site. However, the main challenges in external radiation therapy methods are the efficient tumor treatment with lower doses and lowering the adverse effects on healthy tissue (4, 5). To address this challenge, developing an external method that localizes efficient doses to target tissues while minimizing absorbed radiation to nearby vulnerable healthy tissue is necessary. One of the strategies for reducing the adverse effects of radiotherapy on healthy tissues is using radio-sensitizers. Nanoparticles (NPs) gather close to cancerous cells because of enhanced permeability and retention (EPR). Thus, they can be a suitable radio-sensitizer in advanced therapeutic settings (6). Application of metal-based NPs as radio-sensitizers has gained momentum among researchers (7, 8). Bismuth-derived NPs, e.g., bismuth sulfide (Bi_2S_3), could be used in combination with radiotherapy-based therapeutics in order to induce apoptosis efficiently. Bismuth-based NPs have many advantages, including their reasonable price, high atomic number and adequate photoelectric absorption coefficient (9, 10).

Furthermore, unirradiated cells far from the treated sites, might represent certain features of affected cells, i.e., more mutations, changed apoptosis, and less clonogenic capacity, which is known as radiation-induced bystander effect (RIBE) (11, 12). The transmission of RIBE mainly happens through paracrine effect and *via* cell-cell contact as well as through released soluble factors in the culture medium. Recent updates demonstrated association between RIBE and oxidative stress. Interleukin 1 and 8, tumor necrosis factor, nitric oxide, and transforming growth factor beta 1 were recognized as RIBE factors.

However, it has been demonstrated that the application of NPs in radiation-based treatments altered cytokine secretion profile, ROS production level, gene expression pattern, and bystander signaling effects (13). The effect of radio-sensitizing agents on the bystander responses has been assessed in a few studies (14, 15).

2. Objective

This research aims to assess the RIBE effects after application of bismuth sulfide NPs on MRC-5 and A549 cells, human fetal lung fibroblasts and lung carcinoma cells respectively. In addition, we showed how these NPs affects the therapeutic ratio.

3. Material and Methods

The study protocol complies with the 1975 Declaration of Helsinki's ethical principles. This study was conducted in Zanjan University of Medical Sciences and health services and has been approved by the ethical committee of said university. (IR.ZUMS.REC.1399.414 & 418)

3.1. Cell Culture

A549, lung carcinoma cells, MRC-5 and human fetal lung fibroblasts were cultured in medium as target and bystander cells, respectively. We used Dulbecco's modified Eagle's medium, penicillin/streptomycin 1%, enriched by 10% FBS, at 37 °C in a standard cell-culture incubator.

3.2. Synthesis and Characterization of NPs

In our previous publication we described how to synthesize and characterize BSA-coated Bi_2S_3 (Bi_2S_3@BSA) NPs (9). The fabricated NPs' average hydrodynamic size was 78.9 nm. Before administration, NPs were re-suspended and vortexed for 30 seconds in order to reduce aggregation and agglomeration.

3.3. Irradiation of Cells

When the confluency of A549 cells was about 50%, they were incubated over night with 40 μg. mL^{-1} of Bi_2S_3@BSA NPs. At this cell density, only the soluble factors released in medium provide cell to cell cross-talk. Two different doses of 6 MV photon beam—2 Gy and 8 Gy—were applied to the cultured cells at Valiasr Hospital, Zanjan, Iran. The linear accelerator was Siemens, Germany.

3.4. Medium Transfer

Following irradiation, the cells were incubated for 4 hours in a standard incubator and the medium was transferred based on the Mothersill and Seymour technique (16).

3.5. Viability Assay

To assess the viability of MRC-5 cells after radiation-induced bystander response and the radio-sensitizing efficacy of Bi_2S_3 @BSA NPs on the A549 cells, we used the MTT test. A549 cells were cultured at a

density of 3×10^3 cells/ well in a 96-well plate. The cells were incubated with following concentrations (5, 10, 20, 40, and 80 µg. $mL^{-1)}$ of NPs overnight before radiation. We cultured MRC-5 cells in a 96-well plate (5×10^3 cells/well) and used the ICCM of the target cells to assess the effect of the NPs on bystander response. After incubating the cells for 24 hours, the medium was removed and replaced by 5 mg. mL^{-1} MTT solution. After 4 hours of incubation, the MTT solution was removed and DMSO was added. Finally, the absorbance rate was measured at 570 nm.

3.6. Analysis of Gene Expression

After harvesting the treated cells, their total RNA was extracted using Trizol reagent according to the manufacturer's instructions. (Invitrogen Life Technologies Co., Waltham, MA). Then, synthesis of complementary DNA (cDNA) was done using 2 µg of total RNA from each sample according to the instructions of the cDNA Synthesis kit (Fermentas,USA). The expression levels of *BAX* and *XPA*, the key genes in apoptosis and DNA repair respectively, were measured using quantitative real-time PCR. The primers used in this study were listed in **Table 1**.

3.7. Flow Cytometry Analysis

To assess cell death rate, we performed Annexin V-FITC/ PI labeling technique (eBioscience, USA) according to the manufacturer's protocol for both cell lines.

3.8. Data Analysis

One-way analysis of variance (ANOVA) followed by Tukey's test were performed to analyze the significant differences between groups using SPSS software. The results of $p<0.05$ (n=3) were considered statistically significant. The results were represented as Mean ± SD. (n=3).

4. Results

4.1. Effect of Bi_2S_3@BSA NPs on Radio-Sensitivity and Bystander Response

The viability of MRC-5 and A549 cells after treatment with Bi_2S_3@BSA NPs was evaluated using the MTT test. The results indicated that, at all employed concentrations of Bi_2S_3@BSA NPs, the viability of A549 cells treated with NPs at 2 and 8 Gy(s) were considerably lower than cells that were only radiated. (**Fig. 1A**). This confirms the role of Bi_2S_3@BSA NPs as a radio-sensitizing agent.

The MRC-5 bystander cell's viability incubated in ICCM (derived from 2 Gy irradiated A549 cells cultured with NPs at concentrations > 10 µg. L^{-1}) was significantly less than of MRC-5 cells that received ICCM derived from only irradiated A549 cells ($p<0.05$) (**Fig. 1B**). This difference was insignificant at all concentrations of NPs with a radiation dosage of 8 Gy. This finding was consistent with the MTT assay results and implies that at the higher dose of 8 Gy, NPs were ineffective in enhancing bystander responses in the MRC-5 cells.

4.2. Effect of Bi2S3@BSA NPs on Apoptosis and DNA Repair-Related Genes

Molecular evaluations were performed to investigate the effect of incubation with Bi_2S_3@BSA NPs on

Table 1. The primers used in real time PCR

Name	Oligo sequences
XPA	Forward: AGCAAAGGAAGTCCGACAGG
	Revers: CACACGCTGCTTCTTACTGCTC
Bax	Forward: GGTCTTTTTCCGAGTGGCAGC
	Revers: TGATCAGTTCCGGCACCTTGG
B-actin	Forward: AGCACAGAGCCTCGCCTTT
	Revers: CTCGTCGCCCACATAGGAATC

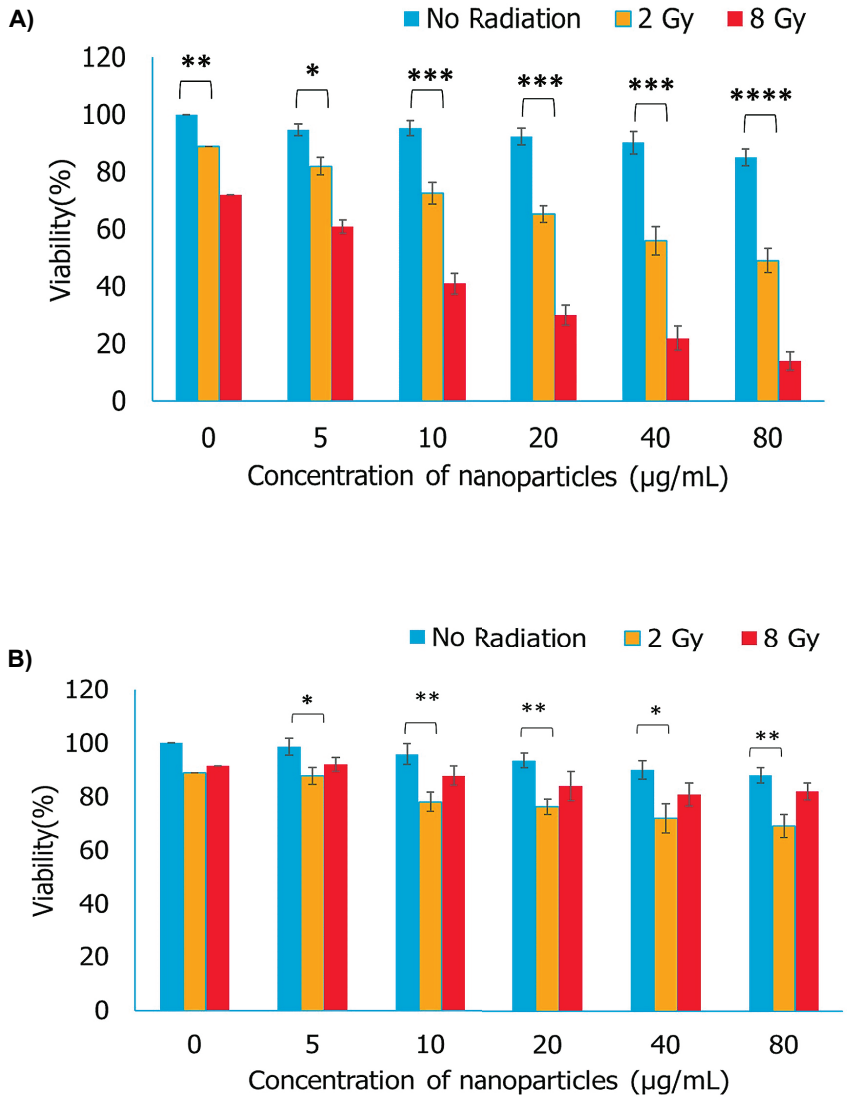

Figure 1. The effect of different concentrations of Bi_2S_3@BSA NPs with and without radiation on the viability of A) A549 target cells and **B)** MRC-5 bystander cells assessed by MTT assay. The signs of *, **, ***, and **** are represented for $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.0001$ respectively.

MRC-5 bystander and A549 target cells. In the presence of Bi_2S_3@BSA NPs, *BAX* and *XPA* genes upregulated after treatment with 2 and 8 Gy in A549 cells ($p<0.05$) (**Fig. 2A**). This data reveals the efficient radio-sensitizing property of the NPs.

As shown in **Figure 2B**, the expressions of the *BAX* and *XPA* after treatment with 2 Gy in MRC-5 bystander cells was up regulated. Interestingly, when radiation exposure and NPs were used simultaneously this up regulation was higher. However, after treatment of cells with 8 Gy, the upregulation of genes diminished and were less than the changes after treatment with 2 Gy. Moreover, *BAX* and *XPA* changes did not show any significant alteration between the combined 8 Gy/NPs and only 8 Gy groups. Additionally, to compare the effect of treatment on the repair and apoptotic pathways, the ratio of *XPA*/*BAX* in the target and the bystander cells were calculated. In MRC-5 cells, the *XPA*/*BAX* ratio between the groups did not change significantly, however this ratio showed a downward trend as the exposure intensity increased in A549 target cells.

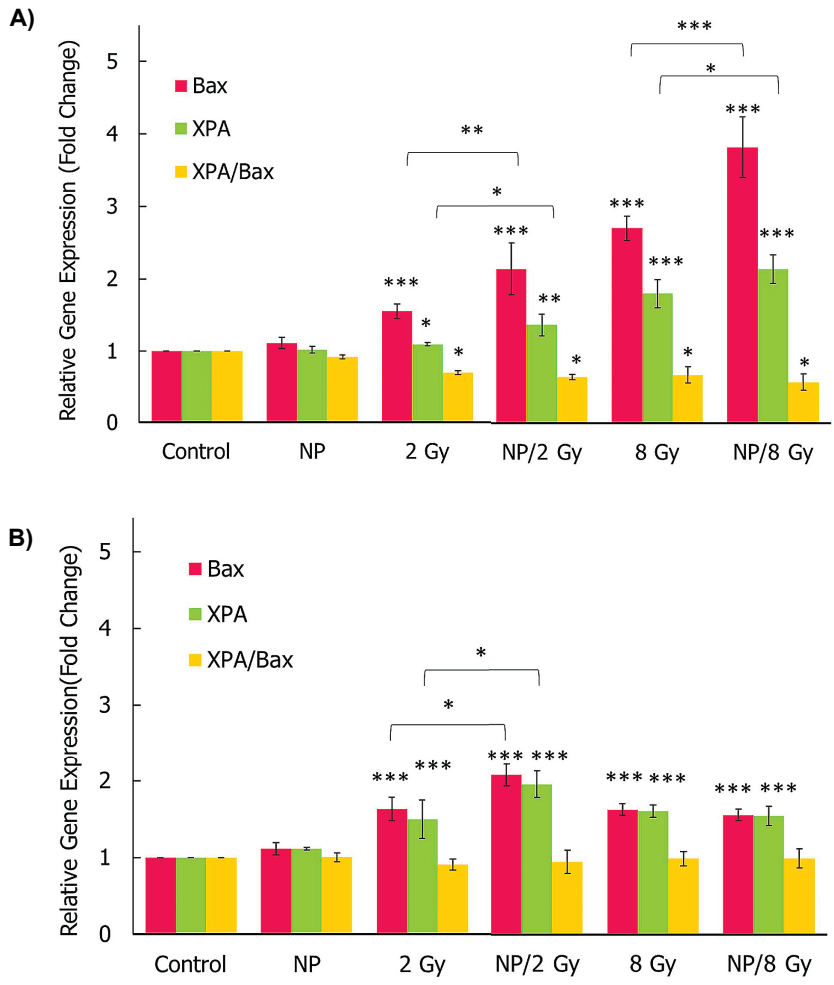

Figure 2. The effect of different treatments on the Bax and XPA expression and their ratio in A) A549 target cells and **B)** MRC-5 bystander cells. The signs of *, **, and ***, are represented for $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ respectively.

4.3. The Cell Death Inducing Effect of Bi_2S_3@BSA NPs

Bi_2S_3@BSA NPs induced cell death in their target cells. These cells and MRC-5 bystander cells were analyzed by Annexin V-FITC/PI labeling technique to determine the proportion of necrotic and apoptotic cells (**Fig. 3**). Analysis of flow cytometry indicated that irradiation in the presence of NPs increased the overall cell death rates in the A549 target cells compared to radiation alone. These alterations highlighted the effectiveness of the bismuth sulfide NPs as a radio-sensitizing substance. To evaluate the bystander signaling of Bi_2S_3@BSA NPs, the rate of cell death in MRC-5 cells incubated with irradiated cell conditioned medium (ICCM) was assessed. Considering the proportion of live cells in MRC-5 cells in different groups, data demonstrated that only at 2 Gy, Bi_2S_3@BSA NPs showed bystander-inducing enhancer effect ($P<0.01$).

5. Discussion

The effects of Bi_2S_3@BSA-NPs as an inducer of bystander responses in cancer cells were studied in this research. Developing novel radio-sensitizers have recently gained momentum but few studies have assessed their impact on the bystander effect (14, 15).

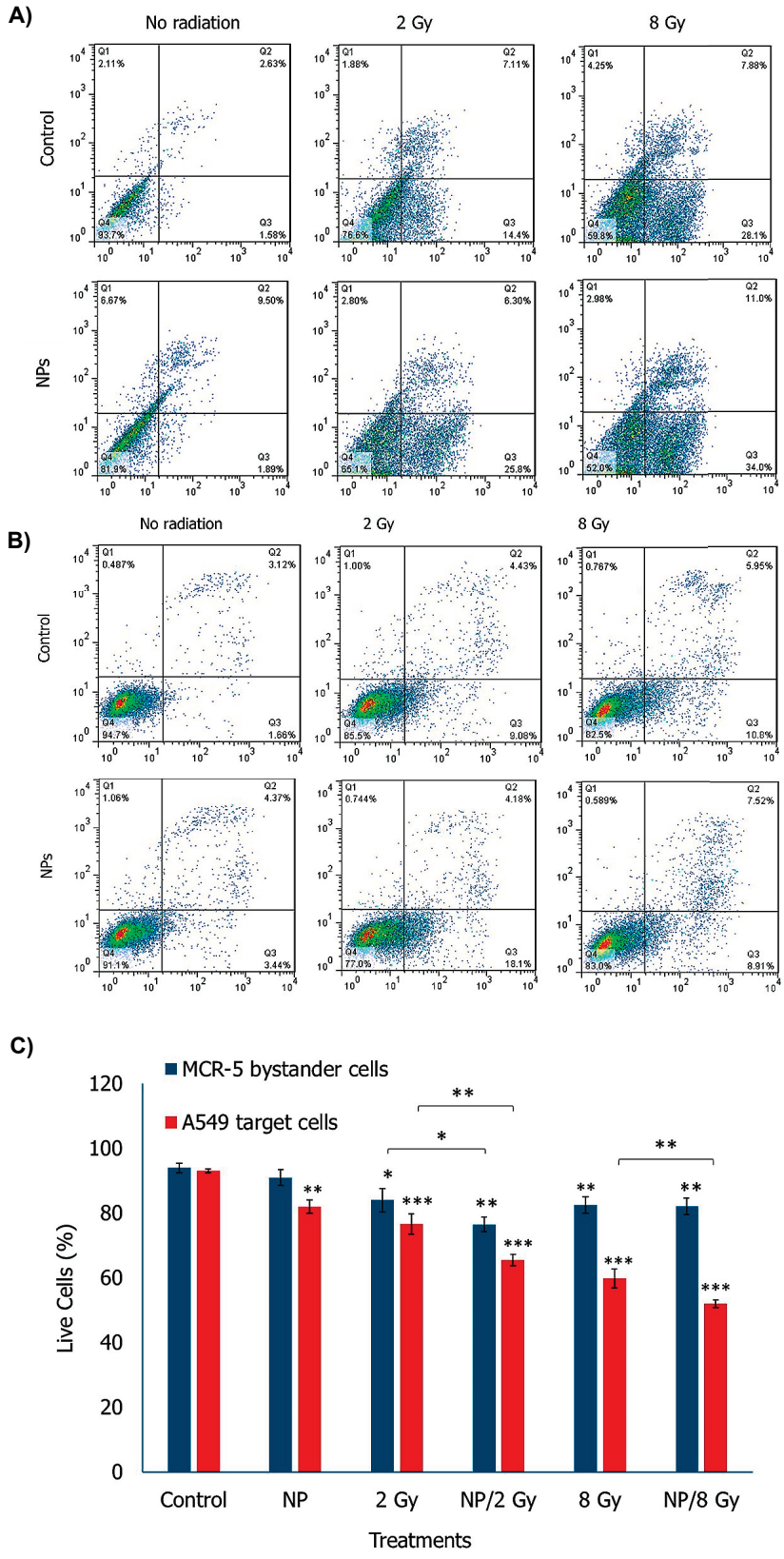

Figure 3. **The effect of different treatments on inducing apoptosis in A)** A549 and **B)** MRC-5 bystander cells. **C)** quantitative representation of live cell population. The signs of *, **, and ***, are represented for $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ respectively.

Application of the Bi_2O_3 NPs did not result in improve-ment in the bystander effect in human fetal osteoblast cells or MCF-7 cells at the doses less than 10 Gy (14). Elevated bystander effect in QUDB cells was reported at 2 Gy of 100 kVp X-rays during application of glucose-coated gold NPs however no effect on RIBE in MCF-7 cells was reported (15). Accordingly, it should be mentioned that the type of cell line as an important factor determining the efficiency of NPs on the bystander response. Based on our results, there was no significant difference in terms of gene expression and cell death rates between the cells who received 8 Gy irradiation in combination with NPs and the cells who received sole radiation. However, this difference was significant between cells that were exposed to 2 Gy and the cells treated with NPs/2Gy. The different patterns of results could propose that at lower radiation doses, the bystander effect might be more effective compared to higher doses. In addition, our data proposes that the bystander effect could be reduced by intensifying the treatment protocol. Interestingly, the expression of *BAX* decreased after treatment with 6 and 8 Gy was recorded. Significant reduction of *XPA* after treatment with 8 Gy was also observed in comparison with the lower doses in the QUDB bystander cells in another study (17). Moreover, conditioned medium-derived from 0.5 and 5 Gy treated bystander cells reduced the bystander cell survival fraction. However, as doses were increased to 10 Gy, the bystander response was abolished. Our data proposed that more exposure induces negative feedback in the bystander cells. Activation of the TGF-β-related signaling derived from the target cells can cause negative feedback (18). Diluting the QUDB-cells-derived ICCM which were exposed to 6 and 8 Gy confirmed our hypothesis. Interestingly, applying 8% conditioned medium from 6 Gy irradiated cells and 6% conditioned medium from 8 Gy irradiated cells led to a higher number of micro-nucleated cells (19). Our data showed that the application of 8 Gy (higher dose) increased viability in bystander cells. Upregulation of *BAX* and the *XPA* genes were declined as well. In addition, bystander responses at 8 Gy did not change in the presence of NPs. Our data was supported by other publications that mentioned negative feedback.

Application of NPs in some radiotherapeutics e.g., stereotactic radiosurgery, IMRT, intraoperative radio-therapy, brachytherapy, and hypo-fractionated protocols suggests a radiation enhancer agent, in which a higher dose per fraction can be applied. This approach appears more useful for cancer patients. Although, more studies need to be conducted to evaluate these finding for different types of radiation, higher doses, (more than 10 Gy), and different cell lines from different cancers.

6. Conclusion

The current research evaluated the impact of using nano-radio-sensitizers on the final outcomes of the radiation-based treatments on lung cancer cells. Our results showed that the application of NPs at higher dose of radiation did not enhance bystander signals. These findings could be considered as promising results since utilizing radio-sensitizing NPs not only enhanced radiation-related effects in irradiated cell but also did not induce damage in the bystander cells receiving bystander signals through ICCM at higher radiation dose. This may be due to negative feedback. This result may propose safety, feasibility, and possible helpful application of NPs in radio-therapeutics.

Conflict of interest

Nothing to declare.

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