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# Bismuth Oxide Nanoparticles Influence on ROS generation by Targeted and Bystander Cells under Proton Beam Irradiation

Raizulnasuha Ab Rashid[1,D], Noor Nabilah Talik Sisin[2,BCD], Nur Hamizah Mohd Zainudin[3,BC], Khairunisak Abdul Razak[4,AB], Moshi Geso[5,AB], Hiroaki Akasaka[6,AB], Ryohei Sasaki[6,A], Takahiro Tominaga[7,B], Wan Nordiana Rahman[8,9,ABEF], Hayato Miura[10,B]

[1]*Centre for Diagnostic Nuclear Imaging, Universiti Putra Malaysia, Selangor, Malaysia*
[2]*Department of Applied Physics, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia*
[3]*School of Medical Imaging, Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia*
[4]*School of Materials and Mineral Resources Engineering, Universiti Sains Malaysia, Pulau Pinang, Malaysia*
[5]*School of Health and Biomedical Sciences, RMIT University, Victoria, Australia*
[6]*Graduate School of Medicine, Kobe University, Kobe, Japan*
[7]*Faculty of Health Sciences, Hiroshima International University, Hiroshima, Japan*
[8]*Nuclear Technology Research Centre, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Selangor, Malaysia*
[9]*Department of Applied Physics, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia*
[10]*Hyogo Ion Beam Medical Centre (HIBMC), Hyogo, Japan*
**Corresponding author: Wan Nordiana Rahman; wnordiana@ukm.edu.my*

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## Abstract

*Introduction:* The goals of radiotherapy to achieve a therapeutic dose for the cancer cells and lower the dose for the surrounding healthy tissues might be possible with highly localized proton beam therapy. The lethal effects can further be increased by using nanomaterials as a radiosensitizer.

*Material and methods*: In this study, the effects of bismuth oxide nanoparticles (BiONPs) in combination with a proton beam on the targeted and bystander cells were investigated by measuring the level of reactive oxygen species (ROS) generated. Human colon carcinoma cells (HCT 116) were incubated with 0.5 mM of BiONPs and then were exposed to 150 MeV proton beams with doses of 2 to 12 Gy at the dose rate of 0.1 Gy/s in a single fraction. ROS was measured for targeted cells after irradiation, and the effect on the bystander cells was measured using irradiated cell-conditioned medium (ICCM) with and without BiONPs.

*Results*: The results show an increase in the ROS generation for irradiated cells treated with BiONPs. Meanwhile in bystander cells, the results indicated the increment in the ROS generation for cells with BiONPs ICCM compared to those with ICCM only. Nevertheless, the population means difference in ROS levels among bystander cells at different incubation times is insignificant ($p<0.05$). The bystander cells treated with ICCM @ 12 Gy also did not significantly differ from unirradiated cells with BiONPs ICCM @ 0 Gy ($p<0.05$).

*Conclusion:* The application of BiONPs as a radiosensitizer in combination with proton beam therapy could improve tumor control with minimal risk of normal tissue complication.

**Keywords:** Bystander, bismuth oxide nanoparticles, reactive oxygen species, proton beams.

## 1. Introduction

Radiotherapy (RT) uses high-energy ionizing radiation to destroy cancer cells directly and may potentially produce genetic alterations that result in cancer cell death[1]. High-intensity radiation is directed at the primary target of cells, deoxyribonucleic acid (DNA), causing genetic material damage and impeding the cell's capacity to divide and multiply. Ionizing radiation can indirectly injure cells by causing free radical formation and damaging cell DNA[2,3]**.** Nanomaterials have been used to boost radiotoxicity to the malignant region while minimizing the biological effect on normal cells to improve the efficacy of cancer treatment[4]. Researchers have conducted an extensive study on the increase of radiation toxicity on targeted tumor cells using radiosensitizer.

Metallic nanoparticle radiosensitizers such as gold nanoparticles (AuNPs), platinum nanoparticles (PtNPs), and iron oxide nanoparticles ($Fe_3O_4$ NPs) have been found as radiosensitizers capable of boosting the effect of radiation on malignant regions by increasing dose deposition in the desired quantity[5-7]. Further-



Authors' contribution:
A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article.

more, as compared to other metals, $Bi_2O_3$ and bismuth-based compounds are among the least poisonous and biologically non-reactive heavy metals, biodegradable, and biocompatible, making them more appropriate for in-vivo applications[8,9]. Bismuth has a greater Z number (Z = 83) than gold (Z = 79), in theory. As a result, it is considered to have much potential as a dose-enhancing agent in radiation therapy[10,11].

In addition, prior studies had shown that bismuth oxide nanoparticles ($Bi_2O_3$ NPs; BiONPs) could operate as a radiosensitizer in RT[12-14]. BiONPs are a low-cost and promising choice for a high-Z radiosensitizer. However, research on its significance in the bystander impact of radiation treatment is sparse[15]. We are not aware of any research that investigates the effects of metallic BiONPs in response to bystander effects from proton beam radiotherapy. The key concern is if the use of NPs in proton beam radiotherapy results in a bystander effect or a non-targeted effect on nearby tissues.

Radiation-induced bystander effects (RIBE) are the reactions of non-irradiated cells to indirect radiation exposure and are harmed by surrounding irradiated cells[16,17]. RIBE responses vary significantly across the cells studied. Depending on the cell lines, radiation dose, experimental end objectives, and experiment time and duration, they might be helpful or dangerous[18]. Detrimental effects because of cellular damages in non-targeted cells might be triggered due to a bystander reaction from targeted cells[19]. The lack of clarity on RIBE processes and the wide range of outcomes from multiple experimental experiments showed that bystander effects research is an exciting subject for debate in radiobiology studies[20].

In general, the destructive action of RIBE on malignant cells may aid in improving radiation efficacy. According to prior research, irradiated cells can emit reactive oxygen species (ROS) into the media, ultimately impacting bystander cells[21]. ROS molecules generated directly from irradiated cells cannot affect bystander cells due to their short half-lives and small diffusion range. These ROS are expected to trigger the formation of long-lived soluble variables and their release into the medium[22,23]. ROS would react with or exceptionally close to its source or the biomolecule that generated it[24]. Following irradiation, a wide range of ROS was produced from diverse sources, which can have a cumulative impact causing DNA damage and the development of signaling pathways for inflammation, apoptosis, cell cycle arrest, aging, and cancer[25]. The current work aimed to investigate the potential of BiONPs as radiosensitizer for proton beam therapy. The cellular condition of bystander effects due to BiONPs application during proton beam irradiation were also quantified through ROS generation.

## 2. Materials and Methods

### 2.1. BiONPs Preparation

As outlined in the previous work, bismuth oxide NPs (BiONPs) were synthesized using the hydrothermal process[26,27]. Then, in 40 ml of distilled water, 2 mMol/L of bismuth (III) nitrate pentahydrate ($Bi(NO_3)_3.5H_2O$, Sigma-Aldrich, U.S.A) and 3 mMol/L of sodium sulfate ($Na_2SO_4$, Sigma-Aldrich, USA) were dissolved and agitated for 45 minutes at room temperature. The solution was then mixed with 18 mMol/L of sodium hydroxide (NaOH) (Sigma-Aldrich, USA) and constantly agitated until yellowish precipitates developed. The solution was then hydrothermally mixed at 60°C. Deionized water and ethanol were used to wash the yellow precipitates. The powders of BiONPs were obtained by air-drying the mixture at 80°C. In the present study, 80 nm of rod-shaped BiONPs were utilized[15,26,27]

### 2.2. Cell Culture Preparation

Human Colon Carcinoma (HCT 116) cell lines were used in the investigation for irradiation. The cell lines were grown in McCoy's 5A medium (Gibco, UK), which was supplemented with 10% fetal bovine serum (FBS) (Gibco, South America) and 1% penicillin-streptomycin antibiotics (Gibco, CA, USA). The cells were cultivated to the 80 to 90 % confluence at 37°C in a humidified environment of 5% $CO_2$ and harvested with 0.25% of Trypsin-EDTA (Gibco, CA, USA).

Targeted cells experiments: HCT 116 cells (1 x $10^4$ cells/well) were seeded in 96 well-plates with McCoy's 5A media mixture, then treated with 0.005 mMol/L of BiONPs for 24 hours before irradiation.

Bystander cells experiment: The 2 x $10^5$ cells/ml cells were seeded in 25$cm^2$ flasks up to 2.965 ml of media and cell suspension. When the cells were 80% confluent, the cells were ready for treatment. 4 ml of fresh media were added into all flasks and 35 µl of 1 mM BiONPs into the treatment flask (0.5%). The cells with BiONPs were incubated for 24 hours before irradiation, similar to the targeted cells experiment.

The medium transfer approach was used in this work to assess the bystander impact generated by proton beam on non-targeted cells[19,28,29]. A human breast cancer (MCF-7) cell line was employed for the subsequent ROS measurement assays. Conditioned medium from irradiated cells was transplanted to MCF-7 cells from bystanders or non-irradiated cells. The cells were maintained at 37°C in a humidified environment of 5% $CO_2$ in Dulbecco's Modified Eagle's Medium (DMEM) (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics (Nacalai Tesque, Kyoto, Japan). The cells were collected using 0.25% Trypsin-EDTA (Gibco, CA, USA) for the subsequent experiments.

### 2.3. Proton Beam Irradiation Set Up

The irradiations were carried out with a proton beam machine (Mitsubishi Electric, Kobe, Japan). Cells were irradiated with a 150 MeV proton beam transmitted via a ridge filter with a Spread-out Bragg Peak (SOBP) width of 6 cm × 6 cm and SOBP depth of 6 cm. Other irradiation parameters are similar to our previous studies[30,31] including field size of 20 cm × 20 cm, source-to-axis distance (SAD) of 100 cm, and dose rate of 0.1 Gy/sec. The flasks and well-plates were placed between a 6 cm solid water phantom on the top and a 10 cm

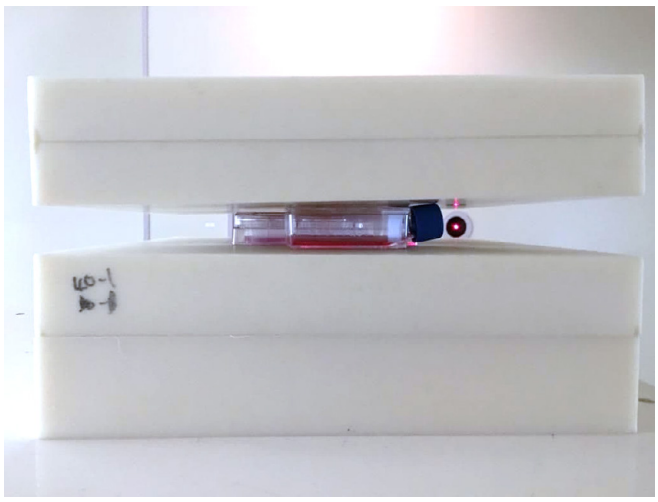

**Figure 1. Proton beam irradiation was set up with 6 cm solid water phantoms on the top and 10 cm solid water phantoms at the bottom of the flasks.**

solid water phantom below the flasks as shown in **Figure 1**. This is to ensure that the cell samples were at depth within the SOBP zone. The delivered radiation doses were in the range of 0 to 12 Gy[15]. **Table 1** shows the treatment components for this study.

**Table 1. Treatment components for the ROS measurement study**

| Treatment | Components |
|---|---|
| Irradiation | 0 (positive control), 2 Gy, and 12 Gy |
| Negative control | Cells + media only |
| ICCM | Cells + media+ irradiation |
| BiONPs ICCM | Cells + media + BiONPs + irradiation |

### 2.4. Harvesting of ICCM for Bystander Cells

The media from the flasks were filtered using 0.22 μm polyether sulfone (PES) membrane filters 2 hours after irradiation to guarantee that no cells were present in the medium[15]. The ICCM were sorted into 15 ml centrifuge tubes according to the treatment components. The ICCM were kept around -20°C for 48 hours, before transferred to -80°C for storage until it was needed for the experiment[32,33].

### 2.5. ROS Measurements of Targeted and Bystander Cells

The dye 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) evaluated intracellular ROS levels in bystander cells (Sigma-Aldrich). The oxidative conversion of cell-permeable DCFH-DA to fluorescent dichlorofluorescein (DCF) was used to identify general intracellular ROS[34].

For the targeted cells' ROS level measurements, the media in the 96 well-plates were directly measured with a Fluoroskan Ascent FL flourescence microplate reader (Thermo Labsystems Inc., Philadelphia, PA, USA) was used with the wavelength of 485 nm excitation and 538 nm emission. The measurements were taken before the irradiation, immediately after irradiation (0 hour), and continue for 1 hour after that.

For the following bystander cells experiment, the frozen ICCM media were thawed at 37°C and introduced to bystander cells, following the procedure described by Jella and colleagues.[35] The MCF-7 cells were seeded into 96-well plates at 1 x $10^5$ cells/well and incubated until 70 to 80% confluency. The cells were rinsed with PBS twice, and 5 μmol/L DCFH-DA dye in buffer was loaded. The plates were incubated in the dark at 37°C for 30 minutes. The cultures were rinsed with Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich) after dye incubation before ICCM and BiONPs ICCM were administered to the cells[15].

A microplate reader (Varioskan Flash, Thermo Scientific) with excitation and emission wavelengths set at 485 and 530 nm, respectively, was employed for the ROS measurement. The fluorescence intensities were determined immediately following the ICCM treatment and at two-hour intervals until the 4$^{th}$ hour, as it was found that the ROS would start to decline at the 5$^{th}$ hour after the ICCM treatment[28]. The amount of fluorescence intensity corresponds to the level of ROS generated in the bystander cells. The mean fluorescence intensity of cells treated with BiONPs ICCM was compared to control cells treated with 0 Gy ICCM. The relative quantity of ROS in targeted and bystander cells was calculated as shown in **Equation 1**.

$$\%\ \text{of ROS} = \frac{\text{DCF treatment}}{\text{DCF control}\left(0\ \text{Gy ICCM of respective hour}\right)} \times 100 \quad (1)$$

### 2.6. Dose Enhancement Factor of ROS levels

The dose enhancement factor (DEF) for the targeted cells was calculated to evaluate the effects of the BiONPs' presence in the media during each irradiation dose. The calculation is shown as in Equation 2:

$$\text{DEF} = \frac{\text{DCF of BiONPs treatment}}{\text{DCF without BiONPs at respective dose}} \times 100 \quad (2)$$

### 2.7. Statistical Tests

All of the tests were carried out in quadruplicate. The data analysis in the measurement of ROS production employed one-way ANOVA followed by the Bonferroni post hoc test. The statistical significance criteria were chosen at $p < 0.05$, the values shown in standard error of the mean (SEM). The analysis was using OriginPro 2018 software (OriginLab Corporation, US).

## 3. Results

### 3.1. Intracellular ROS Generation in Targeted Cells.

The general intracellular ROS level from the HCT 116 cells was measured before irradiation, 0 and 1 hour after irradiation. It was demonstrated that the ROS production at the first reading after irradiation significantly increased but slightly decreased after 1 hour from the previous reading, as shown in **Figure 2**.

Compared to other treatments, the cells treated with BiONPs, and 12 Gy of radiation dose generated a significant amount of ROS. The DEF due to the BiONPs presence at 0 hour were calculated and stated in **Table 2**.

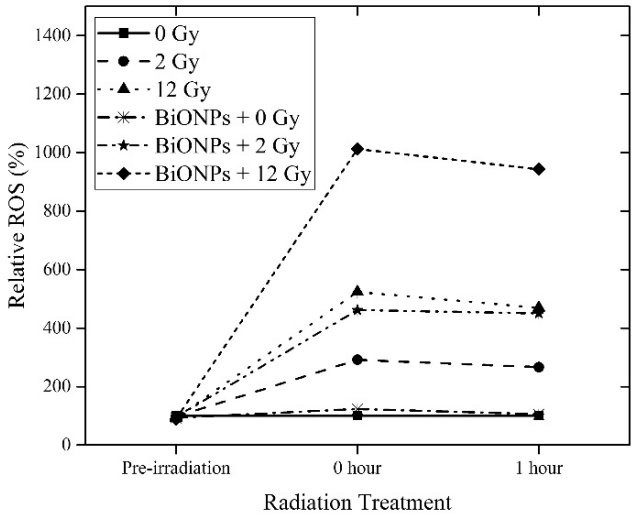


**Figure 2. Percentage of relative ROS level in HCT 116 cells without and with BiONPs, pre-and after irradiations. The values are shown as means ± SEM (n=4). All values of SEM were less than 1.**

**Table 2. Dose enhancement of ROS at 0 hours in targeted cells.**

| Irradiation Doses | DEF values |
|---|---|
| 2 Gy | 1.58 |
| 12 Gy | 1.93 |

### 3.2 ROS Generation in Bystander Cells.

Several analyses could have been conducted to assess the possible radiation effect induced in the bystander cells, including cell viability assay, clonogenic assay, and ROS generation. In this study, the correlation of bystander effect with ROS generation was evaluated, and the result is illustrated in **Figure 3**.

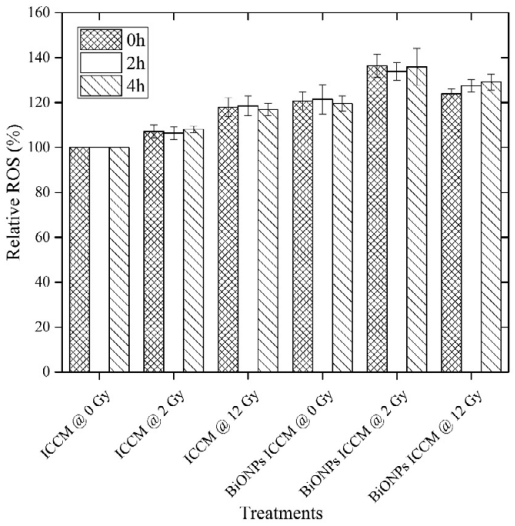


**Figure 3. Percentage of relative ROS level in MCF-7 bystander cells receiving ICCM from 150 MeV proton beam. The values are shown as means ± SEM (n=4).**

The findings showed that the ROS generated from the BiONPs ICCM treatment are higher than the ROS level produced by MCF-7 cells treated with ICCM only. The incubation periods after the ICCM and BiONPs ICCM treatments did not cause any significant difference ($p > 0.05$). For both treatments, the ROS level increased from unirradiated 0 Gy to 2 Gy radiation doses. It is also found that the ROS levels induced by the bystander cells treated with ICCM @ 12 Gy were not significantly different from the ROS by unirradiated BiONPs ICCM @ 0 Gy ($p > 0.05$). On the other hand, the BiONPs ICCM @ 12 Gy induced lower ROS levels than BiONPs ICCM @ 2 Gy.

## 4. Discussion

Generally, intracellular ROS levels would increase after irradiations due to the oxidative stresses induced by the high doses of radiation directly onto the cells[20,36]. In the current study, NPs' presence further amplifies the intracellular ROS production, from 100% to 1012% for the BiONPs + 12 Gy radiation dose treatment. This statement is supported by several works that introduced metallic nanoparticles such as gold NPs on KB mouth carcinoma cells[37], gold NPs *in vivo*[38], iron oxide NPs on MCF-7 breast cancer cells[39], as well as BiONPs on MDA-MB-231 breast cancer cells and NIH/3T3 normal cells[40]. The existence of NPs inside or near the cells would interact with the protons, produce water radiolysis, and induce higher ROS[41,42]. The enhancement of ROS suggested potential radiosensitization during the radiotherapy[43].

According to the findings, the addition of ICCM increased the fluorescence intensity compared to the control group for all radiation doses. Our previous study supported these results, which used a different RT source, HDR brachytherapy, with 0 to 8 Gy of radiation doses on MCF-7 cells[28]. Studies of RIBE on normal cell lines such as osteoblast cells and keratinocytes also showed an increment in ROS level after irradiation with γ-rays[28,35].

Nevertheless, no significant different of intracellular ROS was observed among 0, 2 and 4-hours incubation periods in the bystander cells, without or with BiONPs. This result could be supported by several research. In a study on A375 melanoma cells exposed to non-ionizing ultrasound, cell viabilities of cells with ICCM after 1- and 12-hours incubation were also comparable to the control[44]. Another research on bystander effects of BiONPs with 6 MV photon beam on MCF-7 cells also demonstrated the insignificant different of cell viabilities after 24 hours and 48 hours incubation[29]. Our similar works with BiONPs and brachytherapy found insignificant difference of ROS level at 0-, 1-, 2-, and 4-hours incubations in MCF-7 cells[28]. However, the study also demonstrated significant differences of ROS amounts in hFoB 1.19 cells[28], which indicated that the bystander ROS effects could be cell-types dependent. Research on RIBE for glucose-coated gold NPs and X-rays had observed low and high RIBE in MCF-7 and QUDB cells, respectively[45]. It was also implied that MCF-7 cells could reach

saturation in RIBE at maximum dose of 0.5 Gy[45]. Thus, at higher radiation dose, the production of intracellular ROS was similar regardless the incubation time.

Besides, it was observed that the addition of BiONPs in the media could significantly contribute to the ROS production in the bystander cells without any radiation, similar to the ICCM @ 12 Gy. There is a scarce study on ROS induction as bystander effects from the addition of NPs in ICCM. The mechanisms that significantly caused cellular damage to the bystander cell is postulated due to the ROS generation by the interactions of NP and cell. Previous study reported that Bismuth based NPs may lead to cell death caused of oxidative stress[46]. Moreover NPs highly will induce cell apoptosis and necrosis, where their existence may disturb cell's membrane through different signaling pathways, thus effecting cell environment stability[46].

Nevertheless, a previous work of ICCM with two-dimensional (2D) transition metal dichalcogenide nanosheets demonstrated the 10% elevation of ROS level in THP-1 monocytes bystander cells compared to ICCM alone[47]. Although the observation in this study suggested that the BiONPs are toxic to the non-targeted cells, the RIBE's damaging activity on tumor tissues such as MCF-7 cells may serve to boost radiation efficacy.

On the other hand, ROS generation by bystander cells with the BiONPs ICCM @ 12 Gy was lower than BiONPs ICCM @ 2 Gy. This result revealed that the presence of BiONPs at different doses of radiation gives different reactions to the cells. Research on radio-phototherapy discovered that bismuth NPs could be damaged when irradiated, especially with high doses[48]. It was determined that 12 Gy of X-rays could cause changes to the original bismuth NPs' shape and size[48]. These deviations could weaken the radiosensitization effects [48] and influence the bystander effects. It is also proposed that the high dose induced destructive effect into the bystander emitting cells, hindering the bystander signal[17]. Another study on a new CHW09 anticancer drug also expressed that 12 Gy of X-ray induced a contradiction than the expected anticancer effects on oral cancer cells[49]. Wang and his colleagues inferred that ROS produced dual-functional signaling: DNA damage pathway and protective autophagic pathway[34]. Similar to the present study, it is hypothesized that a high dose of proton radiation to the BiONPs ICCM would render a lowered ROS generation and protect the bystander cells.

Furthermore, the mechanism of RIBE in cancer cells were mediated by several signals, such as micronuclei, mitochondrial dysfunction, autophagy, LC3-II/LC3-I, Beclin-1, AMPK- and NF-kβ protein markers[34,50]. Meanwhile, the addition of silver NPs during the TK6 normal cells' irradiations had shown to induce higher COX2, but low H2AX an 8-OHdG gen expressions[51]. These conditions would stimulate higher RIBE signal transmission. Another study found that RIBE were influenced by ROS level, but did not involve nitric oxide, mitochondrial (mtHsp60), ER stress (BiP, CHOP) and cytoplasmic (cytHsp70) gene expressions in L929 normal cells[19]. Additionally, it was revealed that RIBE in MCF-7 breast cancer cells could be attributed to hormone factors, not cytokines or growth factors[32]. It was inferred that the presence of BiONPs during irradiation in the present study would not prompted similar effects to the silver NPs, due to differences between normal and cancer cells.

## 5. Limitation

While this study focuses on the potential of BiONPs as radiosensitizer for proton beam therapy and their effect in the non-targeted cells, it is important to acknowledge certain limitations, particularly in the absence of direct comparative data against standard nanoparticle-based contrast agents. While this study highlights the imaging potential of BiONPs, certain limitations must be acknowledged. The stability of BiONPs in biological environments remains an open question, as metal-based nanoparticles can aggregate or settle over time, potentially affecting their dispersion and imaging performance. Additionally, the ROS generation efficiency of BiONPs compared to standard nanoparticles is not fully explored, requiring further studies to assess their potential for oxidative stress-related applications. Addressing these limitations through future research will provide a more comprehensive understanding of BiONPs and their suitability for biomedical applications.

## 6. Conclusion

The inclusion of BiONPs as a radiosensitizer in proton therapy had a direct and higher effect on the targeted sites, as well as an indirect effect on the non-targeted malignant region and metastatic cancer cells. The rise in ROS indicates probable radiosensitization during radiotherapy. Although both targeted and bystander cells generated intracellular ROS, the amount of the ROS is significantly different. Therefore, it is critical to understand the processes underlying RIBE responses in different cell lines to minimize harm to healthy tissues around tumors during radiation. Furthermore, understanding the processes behind RIBE responses in irradiated and non-irradiated targeted and non-targeted cells is critical for designing a more effective radiotherapy approach.

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