EFFECT OF NANOPARTICLE SIZE ON RADIOSENSITIZATION EFFECT AND ROS GENERATION IN HUMAN COLON CARCINOMA CELLS (HCT 116) AFTER 150 MEV PROTON BEAM IRRADIATION

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ABSTRACT

Metallic nanoparticles were proven to be a successful radiosensitizer in proton beam therapy. However, the correlation between the particle size and the radiosensitization effects are yet to be elucidated. In this study, human colorectal carcinoma cell line (HCT-116) was treated with PtNDs of different sizes and irradiated with 150 MeV proton beam. The sensitization enhancement ratio (SER) and reactive oxygen species (ROS) generation were quantified. SER analysis reveals that the SER values are proportional to the PtND size, where the largest PtND size available (52 nm) produces SER value of 1.52, while the smallest one (29 nm) possess SER of 1.23. It was observed that the increase in radiation dose does increase the ROS production in all samples. However, the ROS levels in PtND-treated samples express significant reduction compared to untreated samples, and they are diminished with the increase of PtND sizes. The highest ROS value was recorded on 29 nm PtNDs, that possess the lowest SER. 52 nm PtNDs on the other hand, possess the lowest ROS measurement. This work shows that with proper size tuning, PtND can be a potential candidate as an effective radiosensitizer in proton beam therapy. However, the mechanism behind its radiosensitizing effect remains to be elucidated.

ABSTRAK

Nanopartikel logam terbukti menjadi radiosensitizer yang berjaya dalam terapi sinar proton. Walau bagaimanapun, hubungan antara ukuran zarah dan kesan radiosensitisasi masih belum dapat dijelaskan. Dalam kajian ini, garis sel karsinoma kolorektal manusia (HCT-116) dirawat dengan PtNDs dengan saiz yang berbeza dan disinari dengan sinar 150 proton MeV. Nisbah peningkatan kepekaan (SER) dan penjanaan spesies oksigen reaktif (ROS) diukur. Analisis SER menunjukkan bahawa nilai SER sebanding dengan ukuran PtND, di mana ukuran PtND terbesar yang tersedia (52 nm) menghasilkan nilai SER 1.52, sementara yang terkecil (29 nm) memiliki SER 1.23. Telah

diperhatikan bahawa kenaikan dos radiasi meningkatkan pengeluaran ROS pada semua sampel. Walau bagaimanapun, tahap ROS pada sampel yang dirawat PtND menunjukkan penurunan yang signifikan berbanding dengan sampel yang tidak dirawat, dan mereka berkurang dengan peningkatan ukuran PtND. Nilai ROS tertinggi dicatatkan pada 29 nm PtND, yang memiliki SER terendah. 52 nm PtND di sisi lain, mempunyai pengukuran ROS terendah. Karya ini menunjukkan bahawa dengan penyesuaian ukuran yang tepat, PtND dapat menjadi calon potensial sebagai radiosensitizer yang efektif dalam terapi sinar proton. Walau bagaimanapun, mekanisme di sebalik kesan radiosensitizing masih belum dapat dijelaskan.

Keywords: platinum nanoparticle; proton beam therapy; radiosensitization; reactive oxygen species.

INTRODUCTION

Proton beam therapy (PBT) is a powerful tool in delivering highly conformal radiation dose to the deep-seated tumor while sparing the adjacent healthy tissue. The superiority of PBT over conventional photon beam therapy is rooted from the unique characteristic of proton beam interaction within the medium, where most of the accelerated proton particles deposited their kinetic energy at the end of their particle tracks. This phenomenon gives a peak to the dose profile, which is called a Bragg peak. Compared to the photon beam, the surface dose to the patient is significantly lower, while the exit dose is nearly zero, making the PBT a suitable choice in treating the tumor located nearby the sensitive organs (e.g.: eyes, brain). Clinically, the Bragg peak is modulated to give Spread Out Bragg Peak (SOBP) (Fig. 1). This modification ensures better tumor coverage, typically spreading the maximum dose region into a sufficient volume encompassing the treatment area.

Although the PBT entrance dose is considered much lower than photon beam therapy, they still exist. This hinders the possibility to escalate the dose delivered to the tumor, hence limiting the potential efficacy of the treatment. With the hefty cost of mobilizing the treatment, the balance between the costs to benefit of PBT is a point of concern. To address this problem, researchers have developed the radiosensitizers; the materials that can be introduced into the tumor to enhance the radiation dose localization in their vicinity, allowing reduction of dose in the surrounding healthy tissue. Hypothetically, the efficacy of the treatment can be improved using the radiosensitizer, hence reducing the treatment time and cost to achieve the expected treatment result. Radiosensitizers in nano-platform, in particular, has received increasing attention due to their positive toxicological profiles and good localization within the tumors.

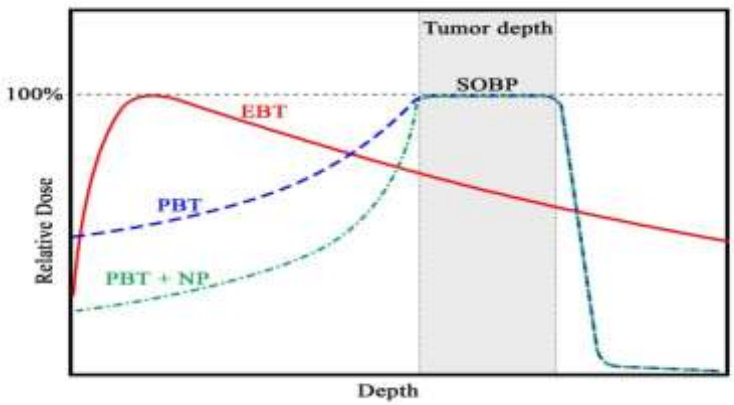


Fig. 1: The dose profile of PBT (dashed line) versus photon EBT (solid line) and hypothetical dose profile of PBT in the presence of nanoparticle radiosensitizers (dashed-dotted line)

Multiple works have reported successful sensitizing effect using nano-radiosensitizers in proton beam therapy. For example, an in vivo study by Kim *et al* [1] has reported a tremendous success in improving the one-year survival of tumor-ridden mice from 11-13% (PBT alone) into 58%-100% (metallic nanoparticle-aided PBT). In the same work, an in vitro experiment was done to verify the effect of reactive oxygen species (ROS) in modulating the efficacy enhanced by the nanoparticles. They found out that the ROS generation in the nanoparticle presence was enhanced compared to the control samples in a dose-dependent manner. This result suggests that the ROS play a role in the mechanism of cell damage by the metallic nanoparticles in PBT.

Recently, Abdul Rashid *et al* [2] has explored the radiosensitization effect of four high-Z nanoparticles (superparamagnetic iron oxide nanoparticles (SPIONs), platinum nanodendrites (PtNDs) and bismuth oxide nanorods (BiNRs)) and their correlation with ROS generation within the cancer cell samples irradiated with PBT. All of the nanoparticles show better cell damage represented by the reduced cell survival in the nanoparticle-treated samples as compared to control samples in PBT. The highest radiosensitization effect was recorded in BiNR-treated samples, and the lowest one was in SPIONs', suggesting that Z of the materials is crucial in manipulating the radiosensitization effects. However, their work does not emphasize on the effect of particle size on the PBT radiosensitization effect. Therefore in this work, we explore the effect of particle size on the PBT radiosensitization effect, as well as their effect on ROS generation to elucidate the mechanism behind their damage to the cells. Platinum was chosen as the material of choice because of their exceptional reputation in the field of cancer therapy (i.e.: cisplatin; chemotherapy) and widely used as a catalyst in the chemical and automotive industry. They can be rapidly fabricated using a chemical reduction method, allowing easy control over their size, which is important in this particular work.

MATERIAL AND METHOD

PtND synthesis and characterization

The PtNDs used in this work were fabricated through chemical reduction method, similar to our previous work [3]. In brief, four sets of potassium tetrachloroplatinate (II) (K2PtCl4) aqueous solution was prepared by dissolving K2PtCl4 crystals into deionized water. The concentration of each solution was set at 5, 10, 15 and 20 mmol/L, in which will yield PtNDs of size 29, 36, 42 and 52 nm respectively at the end of the process.

2 mL of each solution was mixed with 20 mg of Pluronic® F-127 and 2 mL of 88% formic acid before they were immersed in the ultrasonic bath for 12 minutes. The PtNDs produced were washed three times with deionized water and sent for characterization. The PtND concentrations were measured using atomic absorption spectrometry.

Cell culture preparation and protocols

HCT 116 cell line (ATCC® CCL-247™) were cultured in McCoy's 5A medium (Gibco, Life Technologies, UK), supplemented with 10% FBS (Gibco, Life Technologies, South America) and 1% antibiotics (10,000 units / mL penicillin and 10,000 μg / mL streptomycin) (Gibco, Life Technologies, CA, U.S.A). The cells were maintained in a cell culture incubator at 37 °C with 5% CO2 humidified atmosphere until confluent inside 75 cm3 culture flask. Prior usage, the adherent cells were detached using 0.25 % Trypsin-EDTA (Gibco, Life Technologies, CA, U.S.A) and re-suspended in complete medium.

For the radiosensitization effect study, the cells were contained within 200 μL microcentrifuge tubes with the cell density of 1000 cells/tube. Whereas for ROS evaluation, the cell suspension with the 50,000 cells/mL cell density was prepared before they were seeded in 96-well black plates (100 μL/well). The cells contained in the tubes were treated directly in their suspension condition, while the cells in 96-well plates were incubated overnight before they were subjected to treatments.

PtND treatment

For the radiosensitization study, 0.1 mmol/L PtNDs were added to their respective tubes and ready for irradiation. For ROS evaluation, the used media in the 96-well plates were first removed and replaced with new media containing 0.1 mmol/L PtNDs and 9 μmol/L of 2′,7′- dichlorofluorescein diacetate (DCFH-DA) solution (Sigma-Aldrich Pty. Ltd., St Louis, Mo, USA). All samples were prepared in triplicates.

Proton beam irradiation

The samples were irradiated using a proton beam (Mitsubishi Electric, Kobe, Japan) of energy 150 MeV. The irradiation parameters were set as follows: dose rate = 0.1 Gy/sec; SOBP width = 6 cm × 6 cm; SOBP depth = 6 cm; beam field size, FS = 20 cm × 20 cm; source-to-axis distance (SAD) = 100 cm. The samples were sandwiched in between solid water phantoms during the irradiations (top phantom thickness, P_{top}: 6 cm, bottom phantom thickness, P_{bot}: 9 cm) (Fig. 1). The dose prescribed for radiosensitization study was 0, 0.5, 2, 2.5 and 4 Gy, whereas, for ROS study, the dose was prescribed at 0, 3 and 6 Gy. All doses were delivered in a single fraction.

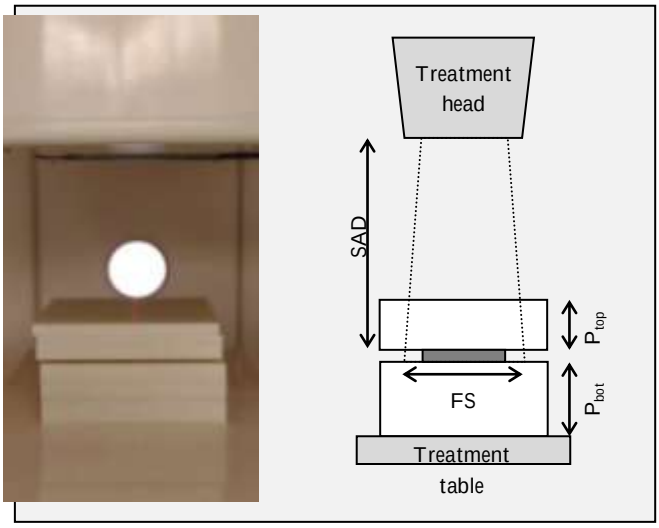


Fig. 2: Proton beam irradiation set up.

Clonogenic assay

The radiosensitization effect of PtNDs was evaluated through the clonogenic assay method. The samples in the irradiated microcentrifuge tubes were extracted and seeded into their respective well in 6-well plates. The cells were incubated for 9 to 12 days until they form colonies containing at least 50 cells each. After that, the cells were fixed using 10% methanol and 20% acetic acid, followed by staining using methylene blue. The number of colonies formed was counted and the survival fractions (SF) were calculated by normalizing the number of colonies in treated samples with the control ones. The SFs were fitted with Linear Quadratic (LQ) model using Origin 2018 software, and the mean inactivation dose (MID) was obtained through the computation of the area under the LQ curves. Then, the SER was quantified using the following equation:

$$\mathrm{SER_{MID}} = \frac{\text{MID of untreated samples}}{\text{MID of PtND treated samples}} \qquad (1)$$

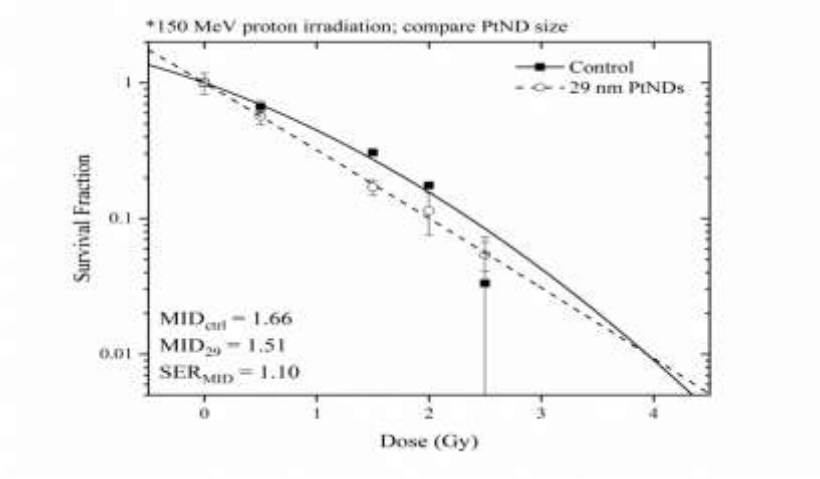
ROS measurement

ROS levels in the samples were assessed using a fluorescent ROS probe, dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich Pty. Ltd., St Louis, Mo, USA). 9 mmol/L of DCFH-DA was added to each sample, and the corresponding fluorescent products were measured using Fluoroskan Ascent FL microplate fluorometer (Thermo Fisher Scientific Oy, Vantaa Finland) with the excitation and emission wavelength set at 485 nm and 538 nm respectively. The measurements were taken before and 2 hours after the irradiations were performed. The ROS generation was calculated from the difference between the ROS level before and after the irradiation. And then, the percentage ratio between the ROS generation of treated samples and control samples were quantified to assess the change in ROS production due to PtND presence.

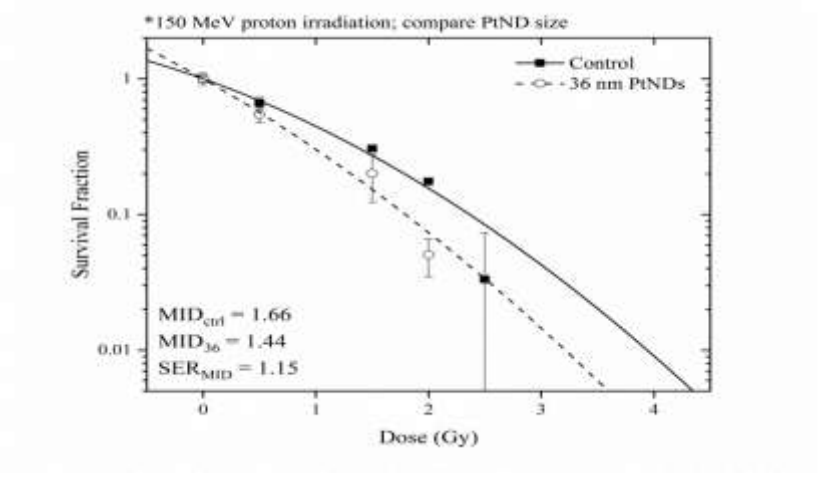
RESULT AND DISCUSSION

Radiosensitization effect of PtNDs

The LQ curves of each PtND and control samples were presented in Fig. 3. Based on the SER quantification, 29 nm PtND gives the lowest radiosensitization effect (SER_{29} = 1.10), followed by 36 nm (SER_{36} = 1.15), 42 nm (SER_{42} = 1.15 nm) and 52 nm (SER_{52} = 1.23) PtNDs.



(a)



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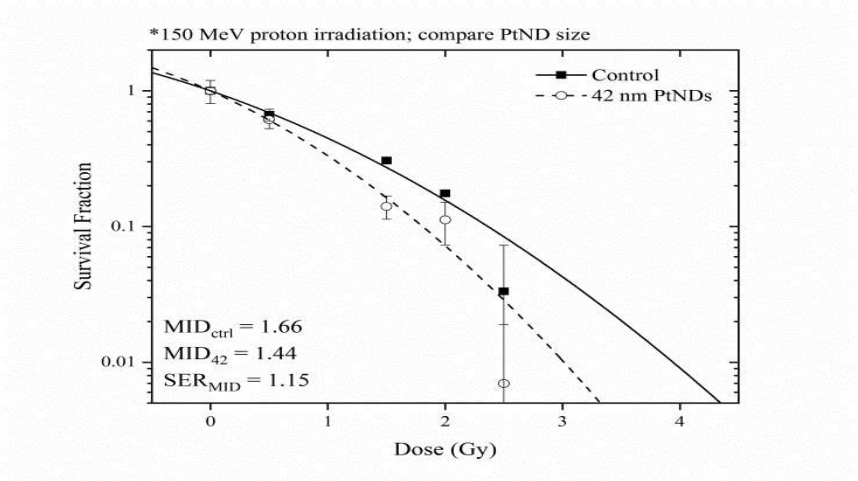
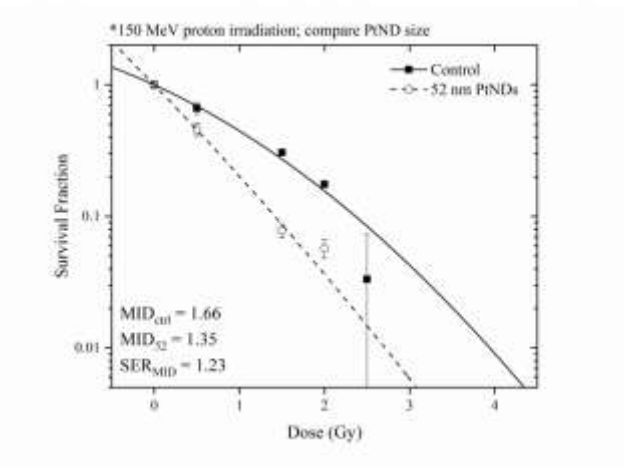


(c)



(d)

Fig. 3: Survival curves of (a) 29 nm, (b) 36 nm, (c) 42 nm and (d) 52 nm PtNDs with the respective control samples.

It was observed that PtNDs were capable of radiosensitizing HCT 116 cells with a magnitude of 10% to 23%, depending on the PtND size. This finding was coherent with previous literatures that show the radiosensitization effect of high-Z NPs in proton beam therapy. For example, Polf *et al* [4] has demonstrated that the gold NPs are capable to enhance the relative biological effectiveness (RBE) of proton radiotherapy in prostate tumour cells. Their group uses gold NPs of size 44 nm and 160 MeV proton beam, which are comparable to our parameters. The gold NPs shows an increase of approximately 15% to 20% of RBE in NP-treated cells compared to control cells. They correlated the decreased cell survivability in gold-treated cells with the enhancement of ionization density due to the interaction of proton particles with gold NPs. The PtNDs used in our study appear to possess comparable enhancement properties as gold NPs used in their work. This phenomenon can be associated with the comparable Z of Pt and Au (78 and 79 respectively), which led us to deduce that the mechanism of radiosensitization by gold and platinum NPs to be analogous.

However, it should be noted that while their work ensures the internalization of gold NPs in their cells, our study on PtND has disregarded the uptake of the NPs by the cells. In our methodology, we used cells in suspension as opposed to the adherent cells. The lack of optimal condition for normal intercellular function, coupled with short term exposure to PtNDs (~2 hours) has made it unlikely for the cells to significantly internalize the particles. Therefore, our result was obtained under the assumption that the PtNDs only resides extracellularly.

To evaluate the effect of NP internalization on radiosensitization effect, Lin *et al* [5] has performed a Monte Carlo simulation involving the gold NP distribution in cellular model. They found out that the internalization of NPs is crucial in enhancing their radiosensitization effect. The SERs are especially high if the NPs are localized within the cellular nuclei, accounting for about 1.81 SER (100 MeV proton beam). In comparison, the SER dropped to 1.018 when the NPs were placed within the cytoplasm, and dropped to 1.001 when the NPs were localized extracellularly. In contrast, the extracellular PtNDs used in our work still give comparable enhancement to the internalized gold NPs in Polf group's work [4]. This led us to hypothesize that PtNDs might possess better radiosensitization effect compared to gold NPs if they can be successfully internalized by cells. In any case, further studies need to be ventured in the future to verify this hypothesis.

ROS generation

It was well-received that ROS plays a dominant role in the mechanism of cell damage in radiotherapy. They are also found to be involved in the formation and progression of several diseases like cancer, diabetes, and neurodegeneration [6]. In brief, a portion the damage done by the radiation to the cells start with the hydrolysis of water molecules, which produces several reactive species such as e_{aq}^-, $HO^\bullet$, $H^\bullet$, H_2 and H_2O_2. They can interact further with the surrounding oxygen, forming superoxide radicals and hydrogen peroxides. These radicals and peroxides can interact with biological materials such as proteins and lipids and induce damages to

the cellular function, ultimately lead to cellular apoptosis [7]. The characterization of the ROS within the medium, hence, can give hindsight in elucidating the pathways of the damage done in the biological system. Several probes have been suggested to measure the ROS in a biological system, with DCFH-DA being the most widely used one nowadays [8]. DCFH-DA works by the oxidation of DCFH anion by the radicals, forming fluorescent DCF molecules that can be detected and measured using a fluorescence microplate reader.

In this work, the ROS level in the cellular environment was measured with respect to the radiation dose and PtND size. The results were presented in Fig. 4. This result is coherent with the previous in-vitro study involving proton beam irradiation [9]. In their work, Giedzinski *et al.* discovered that the ROS level in the 250 MeV proton-irradiated neural precursor cells increases in a dose-dependent manner. Our ROS results in term of dose-dependency correlate well with the cell viability results presented in the previous part, which suggest that the decrease of cell viability with the dose increment can be correlated with the increase of ROS level, disregarding the presence of PtNDs in the medium.

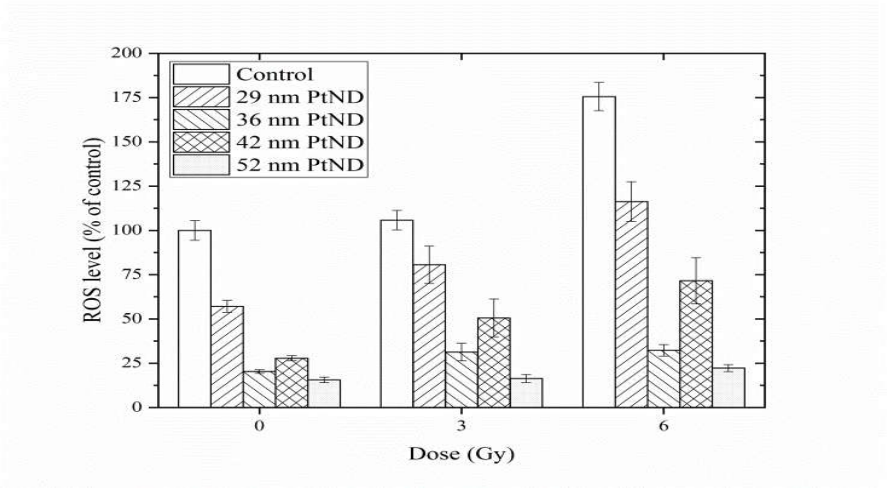


Fig. 4: ROS level in the control samples and the samples containing PtNDs of different sizes.

However, the introduction of PtNDs dose significantly reduces the ROS level in the samples when compared to the control sample at each dose. We can observe in Fig. 4 that the ROS levels in PtND-treated samples are significantly lower than the control sample at each dose. This result is contradictory to the report done by Abdul Rashid *et al* [2], where they show that the ROS level is significantly higher in nanoparticle-treated samples compared to the control one, which is in conjunction with their radiosensitization effects. This result is perplexing because the quantified SER shows that the cell survivals are definitely unfavourable in PtND-treated samples compared to the control samples. While the dose-dependent effects might be explained through the change in ROS level for all samples, the same explanation cannot be applied to discuss the increased radiosensitivity in PtND-treated samples.

We suggest that this phenomenon can be related to two factors. The first one is due to the lack of selectivity of the DCFH-DA probe in identifying specific reactive species. The chemical reactions occurring in the presence of DCFH-DA and its by-products also form a redox cycle, which might overestimate the ROS measurement in the medium. The limitations of DCFH-DA as the ROS probe were reviewed comprehensively by Kalyanaraman *et al* [8] in their literature. While DCFH-DA is a simple and easy assay to use in assessing the oxidative stress within the biological medium, these limitations have made the results to be inconsistent at times. Therefore, a great caution to be addressed in making the conclusion based on this method.

The second factor can be correlated to the catalytic property of metallic nanoparticles. Platinum-based nanomaterials, in particular, have been widely used in the chemical and automotive industry due to their high catalytic property. Kajita *et al* [10] have performed an experiment to verify the scavenging activity of platinum nanoparticles and platinum-gold nanoparticles in the presence of superoxide anion and hydrogen peroxide. They verified that the Pt nanoparticles have a stronger scavenging property compared to Pt-Au nanoparticles. The scavenging activities of both nanoparticle types are dose-dependent. This result leads them to suggest Pt-based

nanoparticles as a potential candidate in combating oxidative-stress diseases. In other work, Watanabe *et al* [11] has shown that Pt nanoparticles are able to scavenge peroxyl radicals and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The scavenging effect on these two radicals has also happened in a dose-dependent manner. These studies proved that platinum nanoparticle is a potent ROS scavenger, which might explain the reduced ROS level within the PtND-treated samples in our work.

The SER results showed significantly high radiosensitization effect portrayed by the PtNDs, especially those with higher particle sizes (Fig. 2). The ROS level, on the other hand, decreased with the particle size increment. The damage to the cells might be attributed to other ROS species that are unable to be probed by DCFH-DA, which are not scavenged by the PtNDs. There is also a possibility that the PtNDs are able to enhance the physical aspect of cell damage which goes beyond the oxidative damage done by the ROS in the irradiated samples. This hypothesis is coherent with the result reported by Jeynes *et al* [12]. They performed an *in silico* and *in vitro* experiment to explore the mechanism of cell damage by gold nanoparticles in photon and proton therapy. In the presence of free radical scavenger (DMSO), there is a reduction in cell killing by photon beam as compared to the samples without DMSO. However, in proton therapy, the difference in cell damage with and without DMSO is less noticeable. They suggest that this result was due to the characteristic of high-LET proton radiation, where the physical aspects of the cellular damage are more pronounced than the chemical and biological mechanism. In any case, further experimentation needs to be ventured to address the raised questions. As a suggestion, we propose that future experiments need to use different ROS probes that possess better specificity in ROS detection. This work also does not take the nanoparticle uptake by the cells into consideration, which has been suggested to have a major contribution in enhancing the radiosensitization effect by the nanoparticles [4]. However, we show that the enhancement by the PtNDs is still significant even without the internalization by the cells. We expect that with proper surface functionalization, PtNDs are able to have better cytotoxicity profiles and cellular uptake, which further enhance their radiosensitization effect in the targeted cells.

CONCLUSION

PtNDs are able to radiosensitize the cancer cells in proton beam therapy by a factor of 1.10 to 1.23 compared to control samples, depending on the nanoparticle size. The ROS level in the PtND treated samples are way lower than the control samples, which suggest that different cell damage mechanism might be involved in the combination of PtNDs treatment and proton therapy. Further research needs to be performed to characterize the actual cell damage mechanisms that engaged in this type of treatment.

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