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RADIATION DOSE ENHANCEMENT EFFECTS OF SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES TO THE T24 BLADDER CANCER CELL LINES IRRADIATED WITH MEGAVOLTAGE PHOTON BEAM RADIOTHERAPY

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ABSTRACT

Therapeutic application of metallic nanoparticles such as gold nanoparticles have been extensively investigated and intriguing finding have been reported. Superparamagnetic iron oxide nanoparticles (SPION) could also potentially have therapeutic properties that can be exploited to enhance radiotherapy outcome. In this study, investigations on the dose enhancement effects inflicted by SPIONs under irradiation with megavoltage photon beam radiotherapy were conducted. T24 human bladder cancer cell lines were pretreated with 1 mMol/L of SPION and irradiated with 6 MV and 10 MV photon beam at different doses.The non-treated cells irradiation was used as a control. Clonogenic assay was performed to determine the cell survival. Linear quadratic (LQ) model are used as fitting curve and does enhancement factors (DEF) were extrapolated from the curves. The cytotoxicity indicated cell growth normally after 72 hours and no long term cytotoxicity effects of SPIONs towards the cells were observed. The dose enhancement effects were observed for both 6 MV and 10 MV photon beam with DEF obtained 1.71 and 2.50, respectively. This reduction of cell colonies growth could be resulted from the interaction that induced free radical and reactive oxygen species (ROS) by megavoltage photon beams. The SPIONs were therefore act as multifunction nanoparticle both in diagnostic agent and radiotherapy as radiation dose enhancer, thus clearly qualified as future theranostic agents.

Keywords: Cancel cell**,** dose enhancement, nanoparticles, photon beam, radiotherapy

INTRODUCTION

Nanoparticles are defined as nanostructures consist of a number of atoms or molecules in nanometer diameter range. The nanoparticles are believed to have specific advantages over conventional drugs in the treatment of diseases and used for various biomedical applications (Mahmoudi et al., 2010). Recently, nanoparticles have been considered as method of providing radiation dose enhancement in tumors (Roeske et al., 2007). In radiotherapy, nanoparticles have potential application as radiosensitizer in enhancing the efficacy of treatment (Kwatra et al., 2013).

Currently, a large number of nanoparticles have been synthesized especially those made from noble metals such as gold (Thakor et al., 2011). In the previous and recent studies, gold nanoparticles (AuNPs) are mostly used as laboratory based clinical diagnostics imaging agent or therapeutic agent. However, to date no clinical application of gold nanoparticles have been approved yet.

SPIONs have been traditionally used for disease imaging and it is recently used for cellular-specifics targeting, drug delivery and multi-modal imaging (Veiseh et al., 2010). SPIONs are magnetic nanoparticles that represent a class of non-invasive imaging agents that have been developed for MRI and currently the only types of nanoparticles that have been approved for clinical study (Mahmoudi et al., 2010). The advantages of SPIONs over AuNPs such as magnetic properties, biocompatibility and cost effectiveness could have benefits radiotherapy especially for hypoxic and radioresistant tumors. Moreover, upon confirmation of their dose enhancing abilities these SPIONs can be considered as theranostic agents aiding in radiotherapy treatments and also use for diagnostics imaging as contrast agents for MRI.

In this study, we investigate the feasibility of SPIONs as potential radiation dose enhancer for megavoltage photon beam radiotherapy. Study were performed *in vitro* using human bladder cancer cells line (T24). Cytotoxic effects of SPIONs without irradiation were also conducted to ensure the effects observed were due to the interaction of radiation and SPIONs.

MATERIALS AND METHODS

Nanoparticles Preparation

SPIONs were synthesised at the Key Centre for Polymers and Colloids, The University of Sydney, Australia, using the procedures reported else where (Bryce et al., 2013; Pham et al., 2015). The 10 nm γ-Fe_2O_3 cores were sterically stabilised by a mixture of short polyacrylamide block (20 monomer units) and polyethylene glycol (17 units) at the molar ratio of 5% to 95%, respectively. The SPIONs were filtered through a sterile 0.22 µm hydrophilic polysulphonic membrane syringe bacterial filter (Sartorius, Goettingen, Germany) before use. The SPIONs dispersion was then diluted with Dulbecco's Phosphate Buffered Saline (D-PBS) (Gibco, Life Technologies, CAL, USA) to achieve the required concentrations.

Cell Culture Protocol

Human bladder cancer cells line (T24) was purchased form ATCC-HTB-4^{TM}. The cells were maintained in RPMI medium supplemented with L-Glutamine, 10% fetal bovine serum (FBS) and 1% antibiotics (10000 units/mL penicillin and 10000 µg/mL streptomycin) (Gibco, Life Technologies). In a humidified environment of 95% air and 5% CO_2 at 37°C, the cells were incubated and subcultivated twice a week.

Cytotoxicity Assay

The study was conducted with different concentration of SPIONs and time of incubation. The cells were tested with SPIONs concentration of 0.25, 0.5, 1.0, 1.25, 1.5, 2.0 mMol/L and control group without SPIONs. The cells were plated in 96-well plates and incubated for 24, 48 and 72 hours in 95% of air and 5% CO_2 humidified atmosphere. The cells viability after incubation were analyzed using PrestoBlue Cell Viability Reagent (Invitrogen, USA. Cat no A13262). The absorbance from the reaction of PrestoBlue with the cells was measured using Bio-Rad Model 680 Microplate Reader.

Cells Irradiation

T24 cells suspension of approximately 1 x 10^4 in counts were prepared in 0.5 mL Eppendorf PCR tube. The cells were prepared with 1 mMol concentration of SPIONs and control cells without SPIONs. The irradiation was performed using 6 MV and 10 MV photon beams from Siemen Primus linear accelerator. The cells were irradiated at 100 cm source to surface distance (SSD) with field size of 10 cm x 10 cm at depth of maximum dose (d_{max}), solid water phantom was placed at a thickness of 15 cm below the samples for backscatter photons.

Clonogenic Assay

The irradiated cells were re-cultured in 6 well plates (SPL Life Science, South Korea) and were left incubated for a week. After incubation, the cells were washed gently with 1 ml Phosphate Buffered Saline (PBS) and were fixed with 500 µL ice cooled methanol. The fixed cells were then stained with crystal violet solution. After the cells were fixed and stained, the visible colonies were counted under a microscope. Survival fractions were calculated for control and cells with SPIONs and are represented by the ratio of colony formation after exposure to radiation to those unexposed to radiation.

Cell Survival Analysis

The cell survivals were analyzed by calculating the survival fraction from the cell colonies according to the equation 1.

$$Survival\ fraction = \frac{Number\ of irradiated\ cell\ colonies}{Number\ of\ control\ cell\ colonies} \quad \text{.........................(1)}$$

The survival fraction for samples with and without SPIONs were then plotted and fitted according to the linear quadratic (LQ) model using OriginPro 8.5 software. The parameters from LQ formula given by the equation 2 were analysed.

$$S = \exp^{-(\alpha D + \beta D2)} \quad \text{.........................(2)}$$

In the equation, S is the the survival fraction and D is the the dose in Gray. The model represented by the linear component [exp (- αD)] and quadratic component [exp (-βD^2)] where α indicate single hit double strand break of two chromosomes and β indicate double hits that induce double strand break of two chromosomes.

The dose enhancement factors (DEF) were extrapolated from the cell survival curves and calculated by taking the ratio of dose that produces 50% of cell survival fraction for control cells to treated cells with SPOINs as depicted in equation 3.

$$DEF_{50} = \frac{D_{50,control}}{D_{50,AuNPs}} \quad \text{..........................(3)}$$

Data Analysis

The survival curve data are presented as the mean ± standard deviation from 3 independent experiments. One-way analysis of variance (ANOVA) was used to compare the percentage of cell survival values among different groups with a 95% confidence interval followed by post-hoc analysis mean comparison using Benferroni's test. All statistical tests were performed using OriginPro 9.2 software.

RESULTS

Cytotoxicity Study

The results of cytotoxic effects of SPIONs are depicted in Figure 1. The cell viability after 24 hours incubation present no significant reduction in cell viability. The cell viability slightly reduced for SPIONs concentration of 0.25 mMol/L and 1 mMol/L. However, the cell viability shows a tiny increment for concentrations of 0.5 mMol/L and 1.5 mMol/L. At 48 hours incubation, the cell viability show no changes from 0 mMol/L to 1.25 mMol/L. There is sudden drop of viability observed at higher concentrations. The results of cells viability at 72 hours incubation are in contrast with the results of 24 hours and 48 hours. The cells viability are increasing for all concentrations. The results indicate cell growth normally after 72 hours and no long term cytotoxicity effects of SPIONs towards the cells.

Quantification of Dose Enhancement Factor (DEF)

Figure 2 and 3 show the effects of different radiation doses of 6 MV and 10 MV X-ray beams alone and in combination with 1 mMol of SPIONs on cell survival fraction. These figures show the potential cellular damage cause by SPIONs with megavoltage photon beams. The DEF values in both of the figures show that the SPIONs induced the enhancement of radiation dose. The shapes of cell survival curves visually shows the reduction of cell survived for cells with SPIONs compare to the control. The DEF value obtained for 6 MV and 10 MV are 1.71 and 2.50, respectively.

Radiobiological parameters of the cell survival curve fitted with LQ model for both 6 MV and 10 MV irradiations are shown in Table 1. The result showed an increment of α (Gy^{-1}) for the irradiation with SPIONs compare to irradiation without SPIONs. While α/β values also shown higher ratio, result the same correlation of SPIONs effects dose enhancement.

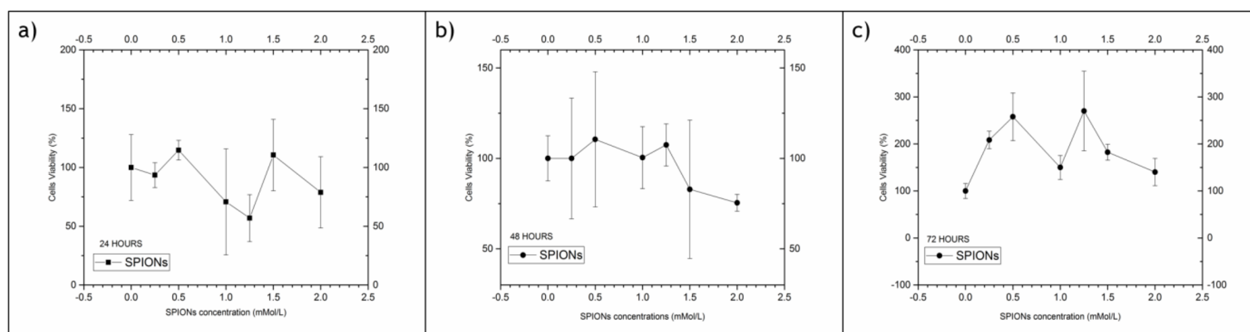

Figure 1: Percentage of cell viability measured for different SPIONs concentration at (a) 24 hours incubation (b) 48 hours incubation (c) 72 hours incubation. Error bar represent standard deviation of the mean of triplicate experiment of each sample

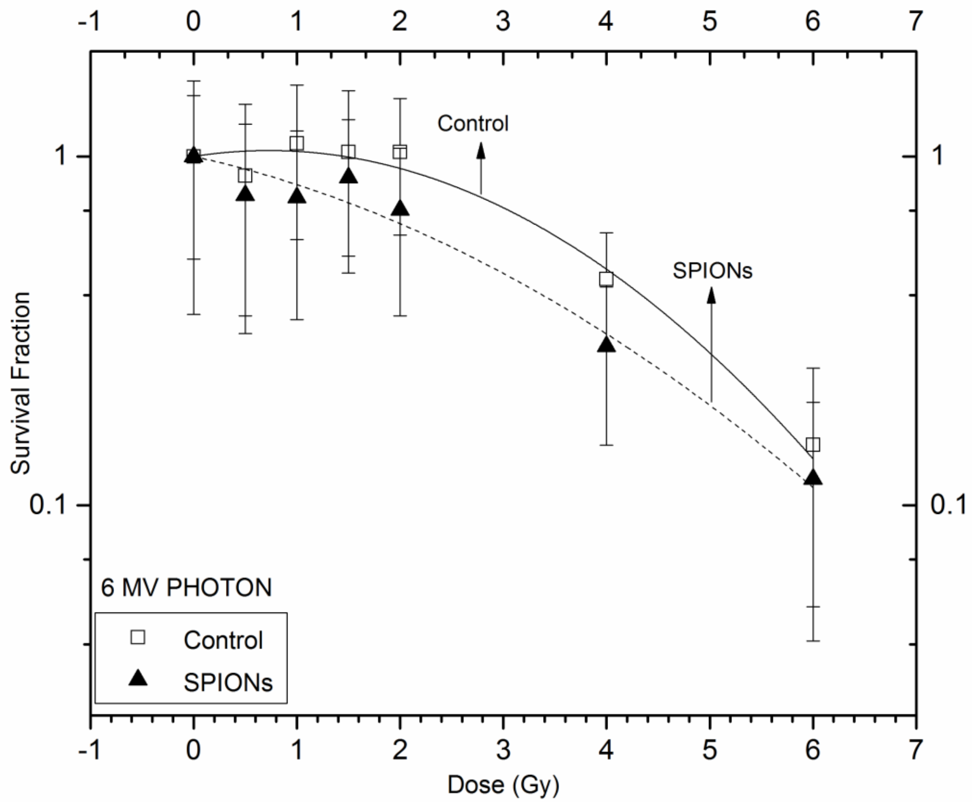

Figure 2: The cell survival curve as a function of dose for cells irradiated with 6 MV photon beams. Error bar represent standard deviation of the mean of triplicate experiment of each sample. The curves were fitted to the data according to LQ model

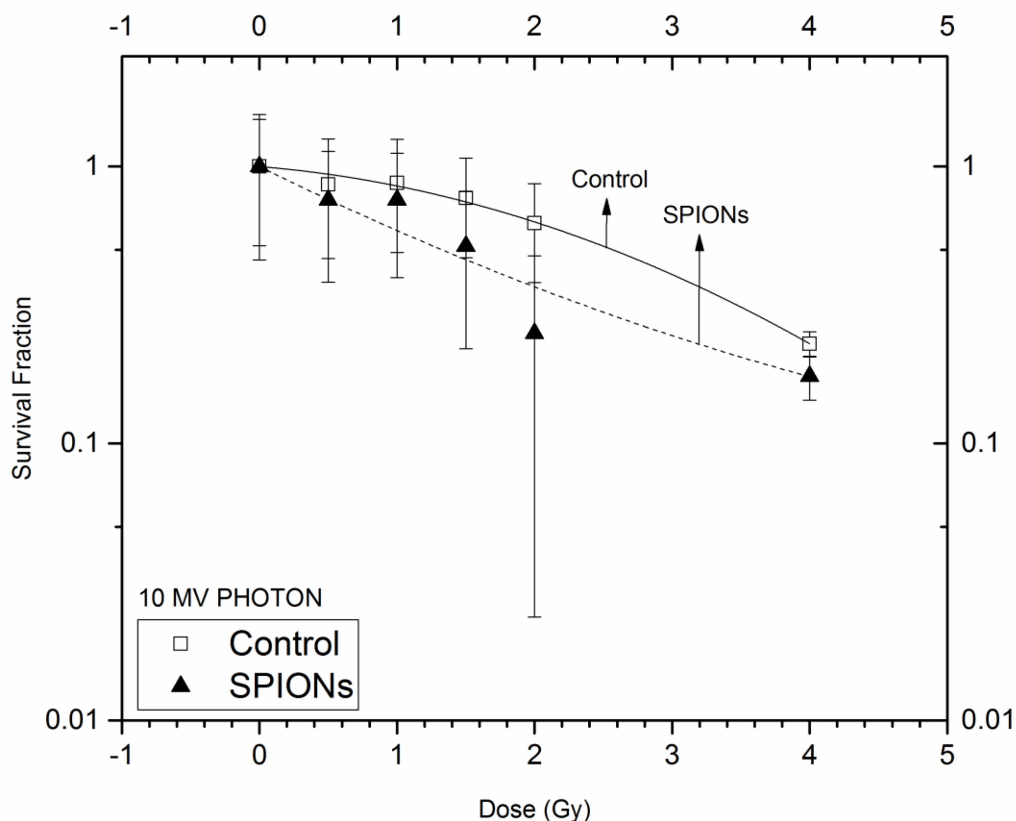

Figure 3: The cell survival curve as a function of dose for cells irradiated with 10 MV photon beams. Error bar represent standard deviation of the mean of triplicate experiment of each sample. The curves were fitted to the data according to LQ model

Table 1: Radiobiological parameters of cell survival curve from Linear Quadratic model

Photon Energy	Test	Alpha (α)	Beta (β)	α/β	DEF
6 MV	Control	-0.11 ± 0.04	0.07 ± 0.10	1.46	1.71
	SPIONs	0.15 ± 0.06	0.04 ± 0.01	4.26	
10 MV	Control	0.09 ± 0.03	0.07 ± 0.01	1.33	2.50
	SPIONs	0.56 ± 0.14	-0.03 ± 0.03	17.83	

DISCUSSION

Cytotoxicity Study

SPIONs toxicity depends on many factors such as particles size, surface coating and cell lines tested. The cytotoxicity study on uncoated magnetic iron oxide nanoparticles (MIONPs) demonstrating different response from variety cell lines. The uncoated MIONPs show toxic effect towards HeLa and RPE cell lines at higher concentration. However, this nanoparticle did not show any acute cytotoxicity at low concentration (Li et al., 2012). Another study proved that the uncoated nanoparticles show less cell viability compared to citrate-coated nanoparticles (Klein et al., 2014). A result obtained by Mahmoudi et al. (2009), indicated that the magnetic nanoparticles give no or low toxicity effects for coated-SPION at high concentration.

The physical and chemical characteristics of nanoparticles may also affect the cell membrane or the intracellular organelles which cause the cells death. Modified iron oxide nanorods show no significant effects while non-modified iron oxide nanorods reduced cell viability at higher concentration (Ghasempour et al., 2015). According to Mahmoudi et al. (2010), modified nanoparticles were safer than non-modified one.

In this study, the cells incubated at 72 hours show more viability at higher concentration compare to control group. The increment occur may due to the iron oxide attribution. Iron is generally able to improve the condition for cell growth. Therefore, the increments of the cell viability at high concentrations could probably due to the abundance of iron in the medium (Ghasempour et al., 2015). However, excessive free Fe ions in exposed tissue may also cause the cytotoxicity and DNA damage. The free Fe ions are imbalanced in the cells homeostasis that will lead to aberrant cellular responses (Elias and Tsuorkas, 2009).

Besides that, the oxidation state of the iron (Fe^{2+} or Fe^{3+}) in the SPIONs could determine the toxicity effects of SPIONs towards the cell lines (Li et al., 2012). The potential of the DNA damage are more likely to be induced by Fe^{3+} ions compared to Fe^{2+} ions. Maghemite (Fe_2O_3) have more significant genotoxicity compared to magnetite (Fe_3O_4) (Singh et al., 2010).

Dose Enhancement Effects

The DEF obtained in this study could be attributed to many factors such as amount of iron diffused into the tumor and concentrations of nanoparticles which play a huge influence on the dose enhancement effects. The probability of the radiation interactions inside the cells with the presence of SPIONs are higher compared to the one without SPIONs. The nanoparticles in the tumor generate secondary radiation interactions which cause the free radical effects and induced the damage to the important organelles such as DNA in nucleus and mitochondria.

The dose enhancement effects could be inflicted by the presence of oxide ion in which increase the formation of oxidative stress. Study on AuNPs dose enhancement effects found that reactive oxygen species (ROS) also play roles in contributing to the dose enhancement and cells damages. However, SPIONs are able to induce much more oxidative stress and increase the ROS stress in cancer cells (Alarifi et al., 2014; Huang et al., 2013). The results of this study show that the presence of oxide ions influenced the dose enhancement effects. This result signifies with the previous finding that employed manganese dioxide nanoparticles as dose enhancer for radiotherapy (Bourzac, 2014). The oxygen levels in the tumors determine the success of the radiation therapy that utilized the oxygen molecule to produce reactive species that damage the cancer cells' DNA (Bourzac, 2014). SPIONs

were also found to induce radiosensitization of human prostate carcinoma cell line (DU145) at egavoltage range of energies (Khoei et al., 2014). Their measurements were in agreement with our results where irradiation with 6 MV photon beams causing radiosensitization effects (Khoei et al., 2014). In our study, we also investigated higher energy of photon beam which is 10 MV and found higher dose enhancement were obtained. The results show that the dose enhancement effects are energy dependent and further study is required to assess these parameters.

CONCLUSIONS

The dose enhancement effects were found to occur for megavoltage photon beams when small concentrations of SPIONs are included in the target. The megavoltage photon beam shows significant dose enhancement up to 2 fold, while electron beam showed. The cells irradiated with SPIONs depicted large α and α/β ratio. Parameters evaluation of the LQ model indicates dose enhancement effects induced by SPIONs which is in agreement with the DEF measured. The dose enhancement effects are dependent on the oxide ions which produce oxidative stress that are important to trigger the cell death and hence increase the enhancement ratio. In conclusion, the results of this research shows clearly that SPIONs can enhance the effects of megavoltage beams which are typically used in radiotherapy treatments. And it is well established that SPIONs are also contrast agent for imaging with MRI. Hence they can be considered as theranostic agents.

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