2017

In vitro investigation of the dose-rate effect on the biological effectiveness of megavoltage X-ray radiation doses

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Abstract

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Keywords

dose-rate, effect, biological, investigation, doses, vitro, radiation, x-ray, megavoltage, effectiveness

Disciplines

Engineering | Science and Technology Studies

Publication Details

Oktaria, S., Lerch, M. L. F., Rosenfeld, A. B., Tehei, M. & Corde, S. (2017). In vitro investigation of the dose-rate effect on the biological effectiveness of megavoltage X-ray radiation doses. Applied Radiation and Isotopes, 128 114-119.

In vitro investigation of the dose-rate effect on the biological effectiveness of megavoltage X-ray radiation doses

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Abstract (80 words)

Radiation therapy is rapidly evolving toward the delivery of higher dose rates to improve cancer treatment. *In vitro* experiments were performed to investigate the response of 9L and MCF-7 cancer cell lines, exposed to 10 MV X-ray radiations. Up to 8 Gy was delivered at a dose-rate of 50 cGy/min compared to 5 Gy/min. The data obtained emphasizes the importance of taking into account not only the physical, but also the radiobiological parameters, when planning a particular cancer treatment.

Keywords: Inverse dose-rate, megavoltage, radiation therapy

1. Introduction

Radiation therapy (RT) is an important modality for many cancer treatments, with over 50% of cancer patients receiving some form of RT as part of their cancer management plan. The ongoing challenge in RT treatment is the controlled delivery of a lethal dose to the tumor whilst minimizing damage to the surrounding normal tissue. Modern radiotherapy techniques such as intensity modulation radiation therapy (IMRT), intensity modulated arc therapy (IMAT) or volumetric-modulated arc therapy (VMAT), and stereotactic radiotherapy (SRT) (Benedict *et al.* 2001; Siochi 1999; Tubiana *et al.* 2000) have improved local tumor control through better precision of the radiation dose delivered. These technologies consequently often deliver more complex treatment fields than conventional techniques.

Clinically, IMRT has become an important modality and it has been widely used in radiotherapy for over 15 years. The capabilities of IMRT have been extensively described in the literature in physical term advantages, such as target coverage conformity, better dose uniformity, and sparing the adjacent normal tissue. These make IMRT superior to conventional or three-dimensional conformal external radiotherapy (3D-CRT) (Cheung 2006; Intensity Modulated Radiation Therapy Collaborative Working Group 2001). However, in IMRT, an increased number of monitor units (MU's) are required, and thus IMRT (beam-on time) generally involves a longer dose delivery time than conventional RT. The radiobiological advantages of IMRT have been extensively debated in the literature (Fowler *et al.* 2004; Ling *et al.* 2010; Lohse *et al.* 2011; Moiseenko *et al.* 2007; Mu *et al.* 2003; Sorensen *et al.* 2011; Wang *et al.* 2003). The suspicion that IMRT could decrease tumor control

due to the increase in the overall treatment time does not have any clinical evidence, and is offset by the advantages linked to the better conformation of physical doses to the target volumes and therefore better sparing of critical organs.

Improvement in clinical outcomes can be achieved by reducing patient intra fraction movements. Recent technical developments have therefore focused on increasing the clinical dose rates to minimize the effect of such movements. Increasing the dose rate also has the added benefit of reducing the overall patient treatment time thus allowing an increase in patient throughput. One recent development is the removal of flattening filters in the linear accelerator heads (flattening filter free (FFF) LINAC configuration), which has proven to be particularly beneficial for IMRT and SRT (Fu *et al.* 2004; Kragl *et al.* 2009; Stathakis *et al.* 2009). The absence of the flattening filter, leading to a significant decrease in the number of MU's for a given photon treatment delivery, has been reported in the literature for both Varian (energies 6 MV and 18 MV) (Stathakis *et al.* 2009) and Elekta (energies 6 MV and 10 MV) (Kragl *et al.* 2009) clinical linear accelerators. It is thus important to, and would be hazardous not to, investigate the biological effectiveness of physical radiation doses delivered with differing dose rates.

Some recent data published related to this has emphasized the radiobiological effect of a high instantaneous dose rate and indicated that there is no effect of the instantaneous dose rate of FFF LINACs on clonogenic cell survival (Sorensen *et al.* 2011). Other data however e.g. Lohse *et al.* (2011), shows that the radiobiological effect of the FFF beam is dependent on the dose per pulse and suggests that this might become a crucial factor that influences cancer cell survival. Ling *et al.* (2010) reviewed the dose rate effect in external beam radiotherapy and concluded that it is the overall beam-on time that determines the tumor cell survival, not the average dose-rate of the linear accelerator (LINAC) nor the instantaneous dose-rates within LINAC pulses. In this work, we are primarily focused on the overall time of irradiation and its influence on the survival of two cell lines with significantly different radiosensitivities.

Dose-rate sparing is usually known to involve a decreased biological response to radiation exposure at a low dose rate compared to a high dose rate, as theoretically predicted by Lajtha *et al.* (1961). On the contrary, Mitchell *et al.* first identified the decrease in survival of HeLa cells irradiated at a low dose rate of 37 cGy/h compared with a high dose rate of 1.54 Gy/h and this was referred to as the "inverse dose-rate effect" (Mitchell *et al.* 1977; Mitchell *et al.* 1979b). Similar studies by Furre *et al.* (1999), also observed an inverse dose-rate effect on NHIK 3025 cells. These data suggest that for some cell lines, a monotonic increase in dose-rate does not produce a similar increase in cell killing. Moreover, in some specific cell lines, increasing the dose rate actually decreases the cell killing effectiveness.

In this article we report on results of *in vitro* experiments in which the biological effect of differing dose-rates for 10 MV X-ray irradiations delivery from a LINAC has been assessed by colony forming assay. The dose is delivered using clinical dose rates of 50 cGy/min compared to a 10-fold higher dose rate of 5 Gy/min. 9L and MCF-7 cell lines were used because they represent a good pre-clinical model of brain and breast tumor tissues respectively. Their intrinsic radiosensitivity is different, with 9L being considered to be more radiation resistant than the MCF-7. To our knowledge, this is the first time that the biological effectiveness of 10 MV X-ray radiation doses on 9L and MCF-7 cell lines has been investigated. The significance in the findings of this study are discussed with regard to observations on the radiosensitivity of the 9L and MCF-7 cells, concepts of radiobiology, and potential implications for new methods of dose delivery (IMRT, IMAT, VMAT, etc.).

2. Material and Methods

2.1. Cell lines

9L, an adherent, fibroblast-like, radio-resistant rat gliosarcoma cell line derived from N-nitrosomethylurea-induced tumor was obtained from the European Collection of Cell Cultures (ECACC). MCF-7 is an adherent, epithelial-like, radiosensitive breast adenocarcinoma cell line established from a 69-year-old female (American Type Culture Collection – ATCC). Both cell lines were tested routinely for mycoplasma contamination. Cells were maintained in exponential growth in DMEM (Invitrogen, AU) with L-Glutamine and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Pen Strep) at 37°C in a humidified incubator with 5% CO_2 in air.

2.2. Irradiation procedures

The irradiations were performed at the radiation oncology department in the Prince of Wales hospital, Randwick, Australia, using an Axesse Elekta LINAC with beam modulator (Elekta AB, Kungstensgatan, Stockholm, Sweden) operated at energy of 10 MV. Single exposure with doses of 1, 2, 3, 5, and 8 Gy were delivered at dose rates of 50 cGy/min and 5 Gy/min.

Experiments were carried out with confluent cultures grown as monolayer in 12.5 cm^2 cell culture flasks with a vented screw cap (BD Falcon) containing 5 mL of DMEM medium and 30 mL of Hanks' balanced salt solution (HBSS). The cells flasks were placed vertically facing the beam and were positioned at a depth of 2.2 cm in solid water to match the D_{max} depth of the 10 MV photon field. An additional thickness of 10 cm of solid water was placed behind the flask to assure adequate scattering conditions. To maintain electronic equilibrium conditions within the flasks during the irradiation, the flasks were also surrounded by solid water slabs. The irradiation field size used for all experiments was 10.4×10.4 cm^2 and the source-to-surface distance (SSD) was 100 cm. Figure 1 shows a schematic diagram of the setup for cellular irradiation. Cell culture flasks were irradiated at room temperature. Unirradiated control samples were kept at room temperature in the control room (i.e at the same condition as the irradiated samples – full of HBSS buffer and placed vertically) while the other samples were irradiated in the LINAC bunker.

2.3. Clonogenic survival

Cell survival was measured by the colony-forming assay, i.e. the ability of a single cell to form colonies *in vitro* (Puck *et al.* 1956). The method used in this study was derived from previous work (Oktaria *et al.* 2015). Both the control and irradiated cells were plated immediately after the irradiation experiments. The medium was removed and the confluent cells were washed gently with DPBS (Ca^{2+} and Mg^{2+} free), then detached using Trypsin-EDTA. The disassociated cells were counted with a haemocytometer and seeded at low densities to achieve approximately 100 colonies after fifteen doubling times into 100-mm tissue culture dishes (BD Falcon) containing 10 mL of complete cell medium (i.e. DMEM containing L-Glutamine and supplemented with 10% (v/v) FBS and 1% (v/v) PenStrep). Depending on the prescribed dose, each experiment involved a maximum of three cells densities with triplicate dishes for each density. The numbers of cells plated per petri dish were determined by preliminary experiments designed in order to determine the radiosensitivity of the cell lines. The cells in petri dishes were then incubated to allow colonies to form at 37°C humidified 5% (v/v) CO_2 cell culture incubator (HERACELL 150i). After fifteen doubling times, the colonies were fixed and stained with a mixture solution of 25% (v/v) crystal violet (Sigma-Aldrich) and 75% (v/v)

ethanol for 5 minutes, washed, and air-dried. Only the numbers of colonies containing more than 50 cells were counted as observed by microscope.

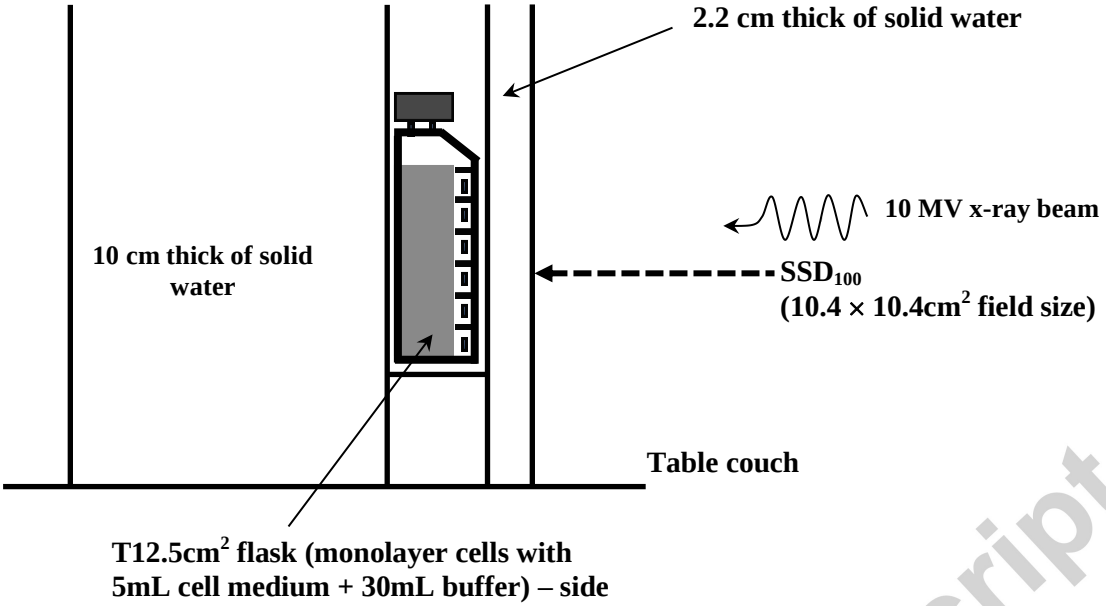

Fig 1. Experimental setup for cellular irradiation.

2.4. Cell cycle analysis by flow cytometry

Measurements of the cell cycle distribution were performed by flow cytometric analysis using propidium iodide (PI) staining. The method used was derived from previous work (Oktaria *et al.* 2015; Vine *et al.* 2007). Cells (2.0 x 10^6) were centrifuged at 1500 rpm for 5 min at 4°C and washed twice with cold phosphate buffer solution (PBS) (Ca^{2+} and Mg^{2+} free) then fixed by drop wise addition of 1mL ice-cold ethanol (70%) and stored at -20°C for up to 7 days before DNA analysis. The ethanol was then removed via a centrifuge process, washed twice with cold PBS. The cells were then stained with a solution containing 40 µg/mL PI, 100 µg/mL RNase A, and PBS (pH 7.4) at 37°C for 1 hour. The stained nuclei were analyzed for DNA-PI fluorescence using a Becton Dickinson fluorescence-activated cell sorting (FACS) flow cytometer (BD LSR II; BD Biosciences, USA). A minimum of 10,000 single cell events was analyzed for each sample in each experiment and at least three independent experiments was performed.

2.5. Data analysis

2.5.1. Plating efficiency and surviving fraction. The plating efficiency (PE) was calculated as the number of surviving colonies divided by the number of cells seeded and the surviving fraction (SF) is the cell survival fraction at dose, D is detailed in (Franken *et al.* 2006) The radiation dose-survival curves were generated by plotting the SF as a function of the dose delivered on a semi-logarithmic scale. The SF expressed as a function of dose (D) curves were fitted using IgorPro software using the linear quadratic (LQ) model $SF(D) = \exp(-\alpha D - \beta D^2)$ where α [Gy^{-1}] and β [Gy^{-2}] are the radiosensitivity parameters (Chadwick *et al.* 1973; Curtis 1986; Fertil *et al.* 1981; 1985; Franken *et al.* 2013).

2.5.2. Flow cytometry data. Cell doublets and aggregates were gated out using a two-parameter histogram of FL2-Area versus FL2-Height. The resulting DNA distribution was then analyzed on the basis of histograms using FACSDiva software (V6.1.3; BD Biosciences, USA) for the proportion of cells in G0/G1, S and G2/M phases of cell cycle. All data were stored by the computer system in a flow cytometer standard (FCS) format.

3. Results

The cell survival curves of the 9L and MCF-7 cells after irradiation with 10 MV X-rays at a dose-rate of 5 Gy/min are plotted on the same graph to allow comparison of the relative radiosensitivities of the two cell lines (Figure 2). From the graph it can be seen that 9L cells are about 2.2 times more resistant than MCF-7 cells. The 10% survival doses (D_{10}) in 9L and MCF-7 are 8 Gy and 3.7 Gy, respectively. Table 1 presents the radiobiological parameters and indicates that the 9L cell line is more radioresistant than MCF-7. These findings are consistent with values published in the literature, which are α=0.24 Gy^{-1}; β=0.029 Gy^{-2} for glioblastomas and α=0.45 Gy^{-1} and β=0.039 Gy^{-2} for adenocarcinoma (Malaise *et al.* 1987).

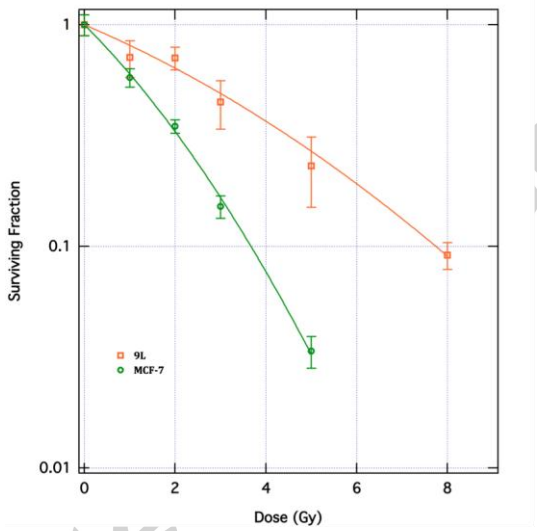

Fig 2. Survival curves of 9L and MCF-7 irradiated at a dose-rate of 5 Gy/min. The data were fitted to the linear-quadratic model. The error bars represent the means ± SEM of at least three independent experiments.

The results for the 10 MV X-rays irradiation experiment at a dose-rate of 50 cGy/min compared to 5 Gy/min on 9L and MCF-7 cells are shown in Figure 3. The reduction of dose-rate appears to have no effect on clonogenic survival of 9L cells (Figure 3A). However, with MCF-7 cells, the lower dose-rate irradiation results in a decreased clonogenic survival (Figure 3B). Analysis of the data using the LQ model is shown in table 1 and reveals that the α value for MCF-7 cells irradiated at 50 cGy/min (0.83±0.01 Gy^{-1}) is larger than the corresponding value when irradiated at 5 Gy/min (0.47±0.03 Gy^{-1}). Hence, the change in the efficacy of treatments was found, as measured quantitatively by a significant reduction of 20% in the necessary absorbed radiation dose in order to achieve the same 10% cell-surviving fraction. The relative biological effectiveness (RBE) was estimated from α and β parameters as the ratio of dose of the reference radiation (dose rate of 5 Gy/min) to that of the test radiation (dose rate of 50 cGy/min) required to reduce the cell survival to 37%. The calculated RBE values were 1 and 1.5 for 9L and MCF-7, respectively.

In an effort to evaluate whether the observed inverse dose-rate effect is linked to the proportion of cells in radioresistant and sensitive phases of the cell cycle, FACS analysis was performed. Under asynchronous confluent conditions, the percentages of cells in G_0/G_1, S, and G_2/M in both cell lines are summarized in Table 2.

(A) (B)

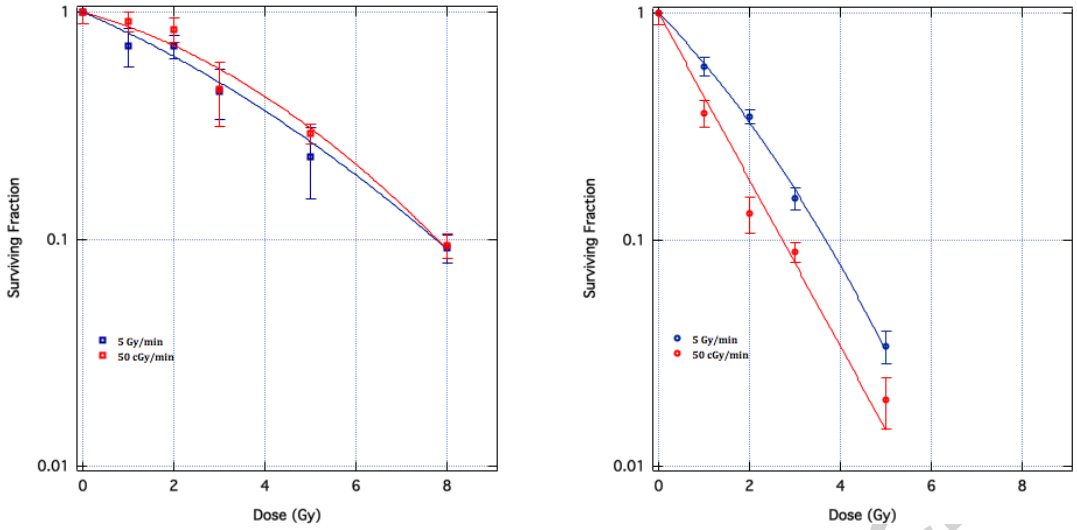

Fig 3. Survival curves of 9L cells (Panel A) and MCF-7 cells (Panel B) irradiated at dose rate of 50 cGy/min vs. dose rate 5 Gy/min. The data were fitted to the linear quadratic model. Each data point represents the means ± SEM of at least three independent experiments (each triplicate).

4. Discussions

Clonogenic assay was used to determine cell reproductive death after treatment with ionizing radiation as described by Puck and Markus (1956) who obtained the first X-ray radiation dose survival curve for irradiated HeLa cells in culture. Since their pioneering work, the intrinsic radiosensitivity of cells derived from human tumors has been the subject of many studies. In the present study, this assay was performed to measure the capability of each cell line to proliferate and produce colonies after irradiation. This relationship can be modeled by a linear-quadratic function of radiation dose.

As is generally true for most glioma cell lines, the 9L cell line is aggressive and less radiosensitive than other rat tumor cell lines (Bencokova *et al.* 2008; Henderson *et al.* 1981). It is the most widely used of all rat brain tumor models for research (Barth 1998) as well as for preclinical model used in brain radiotherapy (Vinchon-Petit *et al.* 2010); it has been utilized in such experiments for more than 30 years (Kimler 1994). The MCF-7 cell line represents a well-adopted pre-clinical model of breast tumor tissue. It has provided the basis of many breast cancer biology studies (Tutt *et al.* 2006) and is relatively more radiosensitive (Matthews *et al.* 1989).

In the present study, we observed no difference between low dose rate 50 cGy/min and high 5 Gy/min dose rate survival curves of the 9L cell line (Figure 3A). Interestingly, we observed an inverse dose rate effect for the MCF-7 cells (Figure 3B), where the lower dose rate of 50 cGy/min induced a 50% increase in RBE compared to that for the reference 5 Gy/min dose rate. The inverse dose rate effect is complemented with a linearization of the cell survival curve when the lower dose rate experiment at 50 cGy/min is performed. The results observed above suggest that the optimum dose rate radiotherapy can be significantly different depending on the tumor cells type.

Several studies have shown that by decreasing the radiation dose rate, recovery processes are allowed to take place during irradiation (i.e. within the first few hours), which usually leads to a reduced biological effect (Hall 1972; Steel 1989). This traditional key characteristic of the dose-rate effect appears to be inversed in this study. The mechanisms underlying the cause of the inverse nature of the dose-rate effect are not well understood, but may be due to several reasons. Possible explanations for this finding are that the effect is dependent on the intrinsic radiosensitivity that varies along the cell cycle at the time of irradiation (Ling *et al.* 1995), cell type (Fertil and Malaise 1981), doubling time (Mitchell *et al.* 1979a), cell age (Biade *et al.* 1997) and more clinically, the histology type (Ramsay *et al.* 1992). Numerous studies have shown that the X-ray induced deficiency in DNA synthesis depends on the cell cycle phase of cells at the time of irradiation (Denekamp 1986; Griffiths *et al.* 1975; Rowley *et al.* 1985). In this study, the underlying mechanism leading to the 20% difference in the efficacy between the treatments was (and still is) unclear. However these positive data warranted the further exploration into identifying a mechanism that manifested as a dose-rate effect on the biological effectiveness of megavoltage x-ray radiation doses on one of the cell lines investigated.

Radiation sensitivity, expressed as loss of reproductive ability, depends on the cell cycle phase in which the cells are exposed. The variation in the cell cycle response to ionizing radiation is believed to be due to the intrinsic radiosensitivity of some human tumor cells (Biade *et al.* 1997). A lower dose rate would then be expected to be more effective if a phase of the cell cycle (i.e. G_2/M-phase) was correlated with the radiosensitivity (Knox *et al.* 1993; Marples 2004; Wilson 2004). Numerous studies have found that plateau-phase cultures are generally more radiosensitive than exponentially growing cells. On the contrary, some studies have observed the development of radioresistance in plateau phase with a V79 cells (Durand *et al.* 1973) and 9L cells (Kimler et al. 1982; Mendonca et al. 1989). However, this condition is minimized in this study as our experiments were carried out with confluent cultures, where the cell number remained constant throughout the irradiation.

The flow cytometry experiments performed as part of this work evaluated whether the observed inverse dose-rate effect is linked to the differences in the intrinsic radiosensitivity between these two cell lines. FACS analysis was performed to analyze the cell cycle distribution and DNA content, indicated that the proportions of cells in the same phase were similar for the two cell lines. Cells are most radiosensitive in phases G_2/M, have intermediate radiosensitivity in G_0/G_1 and are most radioresistant at the S-phase (Tubiana M *et al.* 1990). It is reasonable then to consider that the *in vitro* radiosensitivity of a cell line may be influenced by the proportion of cells in the different phases of the cell cycle. Our flow cytometry results indicate that the phase distribution of the cells most likely does not play a significant role in the observed differences in the radiation sensitivities of the two cell lines.

This study has potential implications for external radiation delivery techniques, where the dose-rate is greatly increased. It is hypothesized that *in vitro* cellular radiosensitivity is correlated with *in vivo* tissue response (Deacon *et al.* 1984). For instance, the α parameter predicts best for intrinsic radiosensitivity of tumor cells irradiated at a dose of 2 Gy (SF2) (Fertil and Malaise 1981; Malaise *et al.* 1987). The clinical implementation of different dose-response parameters on biologically optimized IMRT in breast cancer has been studied by Ferreira *et al.* (2008), which suggest that it can only be maximized by predicting the individual patient radiosensitivity. In general, our study supports the view that individualized radiation therapy may significantly increase the benefits for patients with extreme radiosensitivity or radioresistance.

Since no single molecular factor has been identified that is common to all the cancer cell lines that can explain their radiation sensitivity to radiation dose-rate, an improved understanding of the intrinsic radiosensitivity and damage repair mechanisms should lead to personalized enhanced cancer RT treatments. The results presented in this study may contribute to clinically significant radiobiological effects as the lower dose-rate induced an increased cell killing in MCF-7 cells. It

suggests that some patients with breast cancer would not benefit from the latest technological advances and would better benefit from a dose delivery with a lower dose rate (rather than a higher) for a better treatment outcome.

External beam conformal RT techniques such as 3D-CRT, IMRT, and VMAT have been reviewed for partial breast irradiation (PBI) treatment (Njeh *et al.* 2012) and for malignant glioma of the brain (Wagner *et al.* 2009). There are many papers in the literature showing that VMAT, which enables the delivery of higher effective clinical dose-rates, can be the most efficient treatment option in terms of plan quality and treatment time (better conformal dose distributions with fewer MUs). Although VMAT offers the potential to provide improved target coverage and should be favored because of the shorter treatment time compared to conventional RT techniques, *in vitro* experimental evidence specific to VMAT and its optimization with the biological response of the tumor is lacking. This is in agreement with the observation of McGarry *et al.* (2012) and Butterworth *et al.* (2012) by addressing the difference in the spatio-temporal dose distribution on cell survival following exposure to IMRT and VMAT. Therefore, as highlighted by these authors, there is a need for a definitive radiobiological and clinical aspect for VMAT patient selection. Our data demonstrates that for certain types of cancer cell lines a higher effective dose rate can lead to decreased radiobiological response and therefore a less favorable treatment outcome. Hence, even for radiosensitive patients, a simpler treatment technique (i.e 3D-CRT) is sufficient to maximize the outcome.

5. Conclusions

The data presented in this paper have focused on cell survival following the delivery of 10 MV X-rays radiation doses at a dose-rate of 50 cGy/min compared to a 10-fold higher dose-rate of 5 Gy/min. To date, there has been no literature published that investigates changes in the biological effectiveness of 10 MV X-ray radiation doses on 9L and MCF-7 cell lines with dose-rate. Our *in vitro* experiments show that the lower dose rate induced a larger effect in cell killing in the MCF-7 cell line. This observation emphasizes the importance of taking into account not only the physical dose but also the radiobiological responses when planning a particular cancer treatment. This may help clinicians to individualize patient treatment decisions so as to maximize the efficacy of treatment and hence the clinical outcome of the RT treatment. Moreover, our results may provide important information to predict whether all cancer patients, or perhaps only those with particular tumor types, since we have demonstrated a variation in radiosensitivity, will benefit from advances in the technologies of RT that enable the delivery of X-rays with high dose-rates. Understanding the molecular mechanisms leading to cancer cell death induced by X-ray radiation at different dose rate may provide important information to assist in the further development of novel treatment options in radiation therapy. Future studies that need to be conducted are to evaluate radiation-induced damage and repair process in cancer cells due to dose-rate effect.

Acknowledgements

The authors acknowledge the financial support from the National Health and Medical Research Council (APP1084994).

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Figure Captions:

Fig 1. Experimental setup for cellular irradiation.

Fig 2. Survival curves of 9L and MCF-7 irradiated at a dose-rate of 5 Gy/min. The data were fitted to the linear-quadratic model. The error bars represent the means ± SEM of at least three independent experiments.

Fig 3. Survival curves of 9L cells (Panel A) and MCF-7 cells (Panel B) irradiated at dose rate of 50 cGy/min vs. dose rate 5 Gy/min. The data were fitted to the linear quadratic model. Each data point represents the means ± SEM of at least three independent experiments (each triplicate).

Table 1. Summary of cell survival parameters

Cell line	Dose rate (Gy/min)	α (Gy^{-1})	β (Gy^{-2})
9L	0.5	0.20±0.04	0.013±0.008
	5	0.20±0.06	0.013±0.008
MCF-7	0.5	0.83±0.01	0.00
	5	0.47±0.03	0.040±0.007

Table 2. Summary of cell cycle distribution of unirradiated confluent cell populations

Cell line	G_0/G_1 (%)	S (%)	G_2/M (%)
9L	64.2	12.6	23.2
MCF-7	58.7	13.2	28.1

Highlights

- We examined the biological effect of differing dose-rates for 10 MV from a LINAC.
- Cell survival curves were used to determine the ⟨ and ® values (radiosensitivity).
- A reduction in dose rate has no effect on the survival curve of 9L cells.
- A lower dose rate killed more MCF-7 cells.
- We showed that dose-rate is important in determining the efficacy of IMRT.