AN *IN VITRO* INVESTIGATION OF THE IMPACT OF RADIATION INDUCED BYSTANDER EFFECT ON THE THERAPEUTIC IRRADIATION OF A PROSTATE CANCER CELL LINE.

Thesis by publication submitted for the degree of
Master of Science in Medical Physics
by

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Contents:

Abstract

Introduction.

The aim of radiotherapy, in general, is to deliver a high enough radiation dose to tumour cells to control (and stop) their growth without causing severe complications to surrounding healthy tissues. As a result, it is very important to define a precise irradiation target for radiotherapy treatment. For many years only DNA has been seen as the main target for radiation to cause cellular death in living tissues. In the last decade the fundamental dogma of radiobiology, known as the 'target theory', has been reviewed. The extensive experimental evidence demonstrates that not only cell nucleus but also cellular cytoplasm, membrane, and even neighbouring cells, located outside the radiation field, should be viewed as possible targets for therapeutic ionising radiation.

Methodology.

The research described in this thesis aims to investigate the impact of the non-targeted effects of 6MV x-rays during the radiotherapy. This thesis intends to analyse the published mathematical models which predict occurrence and magnitude of radiation induced bystander effects (RIBEs), with experimental validation of one of these models. The methodology undertaken involved:

- Literature review and development of comprehensive understanding of general concepts of radiation induced bystander effects;
- Establishment of a suitable experimental methodology to investigate these phenomena, in particular radiation induced additional killing, in the application to radiotherapy to PC3 human prostate epithelial adenocarcinoma cell line, including:

    - evaluation of biological characteristics such as population doubling time and plating efficiency;
    - evaluation of radiobiological characteristics such as the dose which kills half of clonogenes (D_{50}), which will be used subsequently as the prescribed dose in the dose cold spot experiment; (in the experiment investigating cell survival in the under-dosed region)
    - determination of suitable biological end-points (such as apoptotic cell death, reduced proliferation rate, clonogenic cell death) following radiation treatment;
    - design of a dose-cold spot experiment to investigate RIBE in a reduced dose region (ie receiving ~80% of the prescribed dose) in freely communicating cells and non-communicating cells;
- Investigation of the extent of non-targeted effects on cell killing in a dose cold spot in human prostate PC3 cancer cell line;
- Analysis of RIBE related models;
- Validation of the published stochastic model that relates absorbed dose to the emission and processing of cell death signals by non-irradiated cells which included:
    - determination of magnitude of medium-borne signals (affecting non-targeted cells) dependence on the radiation doses received by donor cells
    - investigation of donor cell concentration impact on the emission of death signals predicted by the model.

All cell irradiations were performed at the Royal Adelaide Hospital, Radiation Oncology Department using a 6 MV x-ray beam produced by a Varian linear accelerator (Varian, Palo Alto, CA,USA). A clinically applied nominal dose rate of 3 Gy/min was used. Each radiation treatment

was performed at 100 cm from the beam focal spot with 20 x 20 cm^2 radiation field size. The culture dishes were placed on the top of 1.5 cm thick solid water build up sheets. To avoid irradiation through air gaps cells were treated posteriorly with gantry positioned at 180^o. Custom made wax phantoms (for different flask sizes) were used in conjunction with 5 cm thick solid water slab to cover the flask to ensure full scatter conditions. Machine radiation output was routinely checked with Daily QA 3™ device (Sun Nuclear, USA) before each radiation treatment.

The primary research objectives were investigated through a series of research papers.

Results.

The findings and results of the experiments designed and performed in the current work include:

I. Biological characteristics of PC3 cell line such as plating efficiency and population doubling time were found to be 0.60, 48 hours respectively.

II. The fraction of cells surviving the standard clinical daily dose of 2 Gy (SF2) typical of curative radiation protocols was found to be 0.586 (± 0.0279), while the dose that killed half of the clonogen population (D_{50}) was found to be 2.037Gy.

III. Radiosensitivity of PC3 cells differs widely among laboratories - the maximum difference found was 131.58%. This cell line appeared to be very sensitive to the methods used therefore it was important to evaluate D_{50} independently rather than relying on published data.

IV. Apoptotic assay revealed no significant dose dependant early cell deaths until 96 hours after radiation exposure. Following this time the first sizable colonies can be detected by the clonogenic survival assessment. Hence cellular damage in a dose cold spot was assessed by long term survival data which includes all types of radiation induced damages.

V. Cells exposed to a dose cold spot that are freely communicating versus non-communicating cells revealed significant decrease (16.2%) in cells survival presumably due to intercellular communication.

Validation of the stochastic model predicting emission and processing of cell death signals in non-irradiated cells revealed significant decreases in cell survival ($P<0.001$) exposed to irradiated cell condition media (ICCM) derived from donor cells of various concentrations and irradiated with different doses. Dependency of the toxicity of ICCM on the cellular concentration of donor cells was fond to be significant ($p<0.5$) as well.

Conclusion.

For the given cell line under existing growing and treatment conditions the cell survival in the dose cold spot region was significantly lower when under-irradiated cells were in contact with the cells receiving 100% of the prescribed dose compared to the cellular survival obtained from the under-dosed cells, by the same amount of radiation, which were treated separately. Presumably these variations were mainly due to intercellular communication.

Significant reduction in PC3 cell survival after receiving ICCM was observed. Data fitting revealed an exponential decrease in recipient cell survival with the dose received by the ICCM. However the current experiment was not able to identify the associated dose threshold for the reduction in survival from ICCM due to the saturation of the effect at the doses investigated. This can be attributed to either saturation in signal generation due to limited signal potency or saturation in recipient cell responses. It appeared that death signal emission may increase with increasing numbers of radiation hits to a certain target and with increasing number of targets able to emit death

signals. However, the effect saturates when it reaches a specific value in a number of hits or in an amount of critical targets.

The mechanisms behind radiation induced additional killing are not clear yet. Little is known about the types of DNA damage affecting bystander cells. The impact of RIBEs in application to novel radiotherapy treatment techniques, such as intensity modulated radiation therapy and tomotherapy, needs further investigation as they deliver highly conformal doses to tumours, but cover bigger volumes with the low doses where bystander responses are more pronounced.

Incorporation of RIBEs into the research that underpins clinical radiotherapy will result in a shift beyond simple mechanistic models currently used towards a more systems-based approach. It is a difficult task to design a coherent research strategy to investigate the clinical impact of bystander phenomena, given the complex protean nature of it. Any consideration of bystander effects will challenge clinicians' preconceptions concerning the effects of radiation on tumours and normal tissues and therefore disease management.

List of publication by candidate.

Published papers:

Sjostedt, S., and Bezak, E. (2010) "Non-targeted effects of ionising radiation and radiotherapy", Australas Phys Eng Sci Med 33, 219-231.

Sjostedt, S., Bezak, E. (2012) Experimental investigation of the cytotoxicity of medium-borne signals in human prostate cancer cell line", Acta Oncologica,. 04/2012. DOI:10.3109/0284186X.2012.670264

Sjostedt, S., Bezak, E. and Marcu, L. (2012) "Experimental Investigation of the Cell Survival in Dose Cold Spot in Communicating and Non-Communicating Cells", Submitted to Acta Oncologica.

Conference presentations:

- 'Review of the Radiation Induced Bystander Effect and its possible effects in radiotherapy' - Modelling of Tumour meeting 2 (Adelaide, SA, 2008);
- "Experimental investigation of the radiation induced bystander effect and its possible effect on cell survival in dose cold spots." - EPSM ABEC (Christchurch, New Zeeland, 2008);
- "Investigation of the human prostate PC3 cells' survival in a dose cold spot" – Modelling of Tumour meeting 3 (Adelaide, SA, 2010), student paper night (Adelaide, SA, 2010);
- "Experimental investigation of the cell survival in dose cold spots in communicating and non-communicating cells" – EPSM ABEC (Darwin, NT, 2011);
- "Experimental investigation of the cytotoxicity of medium-borne signals in human prostate cancer cell line" – ESTRO 31 (Barcelona, Spain, 2012).

Awards:

The best student poster presentation for “Experimental investigation of the radiation induced bystander effect on cell survival in dose cold spots.” - EPSM ABEC (Christchurch, New Zeeland, 2008).

Statement of Original Authorship.

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Svetlana Sjostedt

Statement of Contribution.

Cell line experiments reported in this thesis were designed and performed by myself. I carried out the background research, however the guidance and scientific advice were frequently provided by my Principal Supervisor A/Prof Eva Bezak.

I was trained and overseen by Dr Tony Cambareri and Dr Fares Al-Ejeh to handle cellular materials and to perform various biological assays.

Cell irradiations for all radiobiological experiments reported in this thesis were carried out by me and Eva Bezak. The beam-on-time parameters for each experimental setup were determined by myself and overlooked by my Principal Supervisor A/Prof Eva Bezak as well as the results scoring and corresponding statistical analysis.

Chapter 2.0 of this thesis contains a version of an article published in Australasian Physical & Engineering Sciences in Medicine as:

Sjostedt, S., and Bezak, E. (2010) "Non-targeted effects of ionising radiation and radiotherapy", Australas Phys Eng Sci Med 33, 219-231.

Sjostedt, S (candidate) performed literature review and wrote the manuscript.

I hereby certify that the statement of contribution is accurate

Signed: .....Date:.....

Bezak, E advised on data interpretation and edited the manuscript.

I hereby certify that the statement of contribution is accurate

Signed: .....Date:.....

Chapter 3.0 of this thesis contains a version of an article submitted to Acta Oncologica as:

Sjostedt, S., Bezak, E. and Marcu, L. (2012) "Experimental Investigation of the Cell Survival in Dose Cold Spot in Communicating and Non-Communicating Cells'.

Sjostedt, S (candidate) performed the experiments and data analysis, interpreted the results and wrote the manuscript.

Certification that the statement of contribution is accurate.

Signed: .....Date:.....

Bezak, E supervised the project, assisted with cells irradiation, advised on data interpretation, and edited the manuscript.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed: .....Date:.....

Marcu, L edited the manuscript.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed: .....Date:.....

Chapter 4.0 of this thesis contains a version of an article published in Acta Oncologica as:

Sjostedt, S., Bezak, E. (2012) Experimental investigation of the cytotoxicity of medium-borne signals in human prostate cancer cell line", Acta Oncologica,. 04/2012; DOI:10.3109/0284186X.2012.670264

Sjostedt,S performed the experiments and data analysis, interpreted the results and wrote the manuscript.

I hereby certify that the statement of contribution is accurate

Signed .....Date:.....

Bezak, E supervised the project, assisted with cells irradiation, advised on data interpretation, and edited the manuscript.

I hereby certify that the statement of contribution is accurate

Signed:…….Date:.....

Acknowledgement.

Completion of this Master project would not have been possible without support and assistance of numerous people. I would like to express my appreciation to my Principal Supervisor A/Prof Eva Bezak for her support, guidance and professional advice provided to me throughout my research project. I am very grateful for her assistance. It has been both a privilege and a pleasure to watch and learn from her.

I would also like to express my appreciation to staff of SA Pathology, Haematology Department for providing us with lab facilities, training me to handle cell line materials and to perform series of biological assays, especially to Dr Tony Cambareri and Mrs Sharon Paton.

Additionally I wish to acknowledge the generous support provided by Dr Fares Al-Ejeh, Senior Research Officer from Experimental Therapeutics Lab, Hanson Institute, for his scientific advice and assistance in performing the dose cold spot radiobiological experiment.

I also would like to express my gratitude to Prof Wayne Tilley and Dr Tina Bianco-Miotto from the Dame Roma Mitchell Cancer Research Laboratories, University of Adelaide and the Hanson Institute, for generously providing us with the cell line materials.

Furthermore, I would like to indicate my appreciation to all colleagues, family members and friends involved in editing and correcting my articles and this thesis, especially to my husband John Sjostedt, my friend Inna Rumokoy and my colleagues Dr Eva Bezak, Dr Justin Sheppard, Dr Loredana Marcu and Mr Joshua Morrees.

Finally my appreciation is extended to my colleagues at Medical Physics Department, Royal Adelaide Hospital, for their support throughout my research project.

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Chapter 1.0.

General Introduction.

1.1. Background to this research project.

Radiation therapy can be used in the treatment of cancer to suppress malignant cell growth, either as a sole treatment or in combination with surgery, chemotherapy and hormone therapy. The scope of radiation therapy includes adjuvant curative treatment to stop cancer cells regrowing, neo-adjuvant treatment to shrink a tumour before the main treatment (usually surgery), or palliative treatment for local disease control and symptomatic relief in cases where cure is not possible [1].

While ionizing radiation is used to destroy abnormal cells; it is also a well-known human carcinogen [2]. Generally it has been assumed that DNA of the nucleus is the main target to destroy a cell and for radiation-induced carcinogenesis. Ionizing radiation interacts with the atom of cell's DNA chain causing the damage. The damage may also result from indirect interaction, where radiation interacts with surrounding water molecules to form free radicals, which then damage the cell DNA. In the common forms of external beam radiotherapy using X-rays, most of radiation induced damage is indirect, through free radical formation and results in cell death, sterilization, cytogenetic changes, apoptosis, mutagenesis, or carcinogenesis [3, 4]. At relatively high doses above 50 millisievert, the cancer risk can be predicted based on the cancer incidence among the Japanese atomic bomb survivors [5] . Consequently at lower doses the deleterious effect of radiation is expected to decline proportionally as fewer cells are likely to be damaged directly or via water radiolysis products.

In yearly 1990s' a third mechanism of radiation damage, termed as 'radiation-induced bystander effect' (RIBE) was observed. The wealth of evidence [6-10] accumulated over the two decades has indicated that both extranuclear and extracellular targeting may play an important role in triggering

the biological responses to ionizing radiation. A major paradigm shift in radiation biology has resulted from work that involved observing the effect of agents and signals emitted by irradiated cells to non-irradiated cells. RIBEs may manifest themselves in various forms, ranging from reduced cell survival, cytogenetic damage, apoptosis enhancement, delayed genomic instability, cell cycle delay, micronucleus formation, delayed mutations and changes in gene expression in neighbouring non-irradiated cells [11-14]. Based on published literature, RIBE has been mainly observed in *vitro* experiments using very low doses (mGy; cGy) of alpha particles [6]. There have also been *in vitro* RIBE experiments performed using x-rays and gamma rays at doses used for conventional radiotherapy and lower[15]. The underlying RIBE mechanisms are complex and still poorly understood. However, it is believed that molecular signalling released from irradiated cells induces various signalling pathways in non-irradiated neighbouring cells, resulting in the cellular responses listed above. The signals transduction may be transmitted through gap junction intercellular channels or extracellular environment through a medium transfer mechanism[16]. The nature of these signalling molecules is diverse and still not conclusively established. However studies such as those by Lyng *et al* [17-19] have demonstrated rapid calcium induction, increase in reactive oxygen species and loss of mitochondrial membrane potential in cells receiving culture medium collected from various generations of cells post exposure.

Maguire et al [20] demonstrated an increase in cell sparing of 15% after they received a priming dose before a subsequent challenge dose. They postulated that a small priming radiation dose (high enough to cause damage) results in the activation of repair mechanisms. This in turn results in the accumulation of various repair proteins at the site of damage, which aids to reduce subsequent damage as a result of the challenge dose [21].

It seems that RIBE may have an important clinical implication for assessment of health risks associated with radiation exposure and also has the potential to reassess radiation damage models

currently used in radiotherapy. Hypothetically, RIBE may result in formation of whole-body or localized side effects in tissues beyond the irradiation field in radiotherapy as well as in low-dose radiological and radioisotope diagnostics. Factors emitted by directly irradiated cells may result in increased risk of genetic instability, which can lead to mutation and secondary cancer induction [22]. In radiotherapy, RIBE may be a potentially harmful or beneficial event. Increase in damage to tumour cells not directly hit by radiation or induction of tumour cell differentiation may contribute substantially to achieve better tumour control by fewer cancers cells killed. If, however, a molecular species secreted by abnormal irradiated cells damage neighbouring healthy cells, it could lead to either early or late responses in these cells such as increased toxicity in normal tissue or increased probability of secondary cancer induction. This is especially important in modern radiotherapy, as conventional 3D conformal radiation therapy (3D-CRT) and intensity-modulated radiation therapy (IMRT) are aimed at diminishing the radiation dose and associated damage in normal tissues. Thus clinical implications of RIBE for radiotherapeutic outcomes are still under investigation.

1.2. Research aim and objectives.

The main aim of this research was to investigate the possible impact of communication between irradiated and non-irradiated cells during external beam radiation therapy. All work undertaken in this project concentrated on three main area of this study. These include:

- A literature review of non-targeted effects of ionising radiation (emphasising on the possible application in radiotherapy);
- A series of in vitro experiments using PC3 prostate cancer cell line. These experiments were design to investigate the impact of RIBE the survival in PC3 cell when exposed to a dose cold spot;

- Overview of the currently proposed RIBE mathematical models. The experimental validation of a microdosimetry based model, that predicts the magnitude of emitted death signals by donor cells and evaluates the probability that recipient non-irradiated cells will survive these signals.

The primary research objectives for this MSc project were investigated through a series of scientific papers centred on these key research goals.

1.3. Research methodology.

Selection of suitable methodology that adequately covers and addresses the specific aims and objectives listed above was essential. The process of progressing from the initial problem formulation to the final task involved several iterations prior to achieving the specified objectives.

The overall methodology involved the following stages:

- An overview and development of a comprehensive understanding of general concepts of RIBEs currently proposed in a literature such as the underlying mechanisms of signal induction and propagation.
- Summary of experimental approaches and biological end points used to investigate RIBE.
- Experimental investigation of the biological properties, namely population doubling time and cell plating efficiency (CPE), of the PC3 human prostate cancer cell line.
- Estimation of clonogenic content including radiobiological parameters of PC3 cell line after radiation treatment with 6 MV x-rays beam. These parameters were;
    - D50, the dose which kills half of the clonogenes population and;
    - SF2, the fraction of cells surviving the standard clinical daily radiation dose of 2 Gy.

- Experimental investigation of apoptotic cell deaths and proliferation rate of PC3 cell line to determine whether these biological endpoints can be used to indicate RIBE in dose cold spot and media transfer experiments.
- Determination of the beam-on-time parameters for each experimental setup involved cell irradiation.
- Investigation of the PC3 cell survival in a dose cold spot. This involved assessment of radiation induced cellular damage in a 20% lower dose region, compared to the cell survival receiving 100% of the prescribed dose (2 Gy) in the experimental set-ups when under-irradiated shielded cells were either in direct contact with cells receiving 2 Gy or irradiated separately.
- Validation of the RIBE microdosimetry based model proposed by Stewart *et al* [23]. In this thesis this part of experimental work is referred to as the 'media transfer experiment'.

More detailed discussion of the research methodologies developed in the current work is described in the following Chapter 3 and 4.

1.4. Thesis outline.

The goal of this research project was to overview, analyse and develop experimental methodologies to investigate the impact of non-targeted effects of ionizing radiation such as radiation induced additional killing in external beam radiation therapy (EBRT).

Chapter 2 of this thesis contains the literature review of basic concepts of radiation induced bystander phenomena. It summarises the endpoints observed in non-targeted cells. It discusses the possible underlying mechanisms and the key molecules involved in signal induction and transduction. Additionally, experimental approaches used to investigate RIBE including designs of

biological assays and radiation setups are reviewed. Chapter 2 also summarises several mathematical models currently proposed in an attempt to quantify RIBE. The main emphasis of this chapter was to analyse and highlight the potential impact of the bystander phenomena in radiotherapy. The review work was used by author to develop and design experiments to investigate the aim of this thesis.

Chapter 3 contains the investigation of the impact of radiation induced bystander killing on EBRT single fraction treatment in *vitro*. This was achieved by assessing radiation induced cellular damage in a 20% lower dose region. The under-dosed cell survival was compared to the survival received 100% of the prescribed dose (2 Gy) in the experimental set-ups when under-irradiated shielded cells were either in direct contact with cells receiving 2 Gy or irradiated separately. The author did not aim to differentiate between possible scenarios of inter-intra-cellular communication for the cells in direct contact but considered all possible mechanisms of cellular signaling involved.

Chapter 4 contains the analysis of the microdosimetry based model proposed by Stewart *et al* [23]. This model predicts the magnitude of emitted death signals released by irradiated donor cells into growth media and evaluates the probability of recipient non-irradiated cells of surviving after being exposure to these signals. According to this model the fraction of non-irradiated recipient cells, surviving transfer into the irradiated cell condition medium (ICCM), decreases exponentially with increase in the signal intensity or the number of irradiated donor cells.

Chapter 4 also describes the experimental work conducted to validate this model by investigation of the toxicity of ICCM which may depend on the absorbed dose received by donor cells and on the cellular concentration of donor cells releasing toxic medium-borne signals due to radiation hits.

Chapter 5 contains general conclusions on why RIBE and cell communication in general need to be investigated and how the radiotherapy treatment techniques can be altered by developing new strategies to modulate beneficial and detrimental bystander responses in tumour and normal tissues.

Chapter 2.0

Non-targeted effects of ionising radiation and radiotherapy: review.

A version of this chapter with content formatted for publication has been published as: *Sjostedt, S., and Bezak, E. (2010)'Non-targeted effects of ionising radiation and radiotherapy", Australas Phys Eng Sci Med 33, 219-231.*

2.1. Abstract.

Modern radiobiology is undergoing rapid change due to new discoveries contradicting the target concept which is currently used to predict dose-response relationships. Thus relatively recently discovered radiation-induced bystander effects (RIBEs), that include additional death, mutation and radio-adaptation in non-irradiated cells, change our understanding of the target concept and broadens its boundaries. This can be significant from a radioprotection point of view and also has the potential to reassess radiation damage models currently used in radiotherapy. This article reviews briefly the general concepts of RIBEs such as the proposed underlying mechanisms of signal induction and propagation, experimental approaches and biological end points used to investigate these phenomena. It also summarises several mathematical models currently proposed in an attempt to quantify RIBE. The main emphasis of this article is to review and highlight the potential impact of the bystander phenomena in radiotherapy.

2.2. Introduction.

Ionising radiation has been used to treat cancer for nearly 110 years [24]. The ability to use radiation effectively, avoiding any complications, is most desirable; however this cannot be achieved easily due to the complicated nature of the interaction processes between biological objects and ionising radiation. Biological responses to radiotherapy treatment are complex multi-staged processes leading to tumour cell death or sterilisation after administrating a sufficient amount of energy. The subject of sufficiency was extensively studied by scientists and practitioners from different fields such as physics, medicine, biology and genetics and led to the development of radiation biological research, one objective of which is to predict the relationships between energy deposition in cells and the probability of cell survival. In the currently accepted concept of the cell killing mechanism the cell nucleus – and DNA in particular – is viewed as the main target which must be damaged to destroy a cell [25].

In the last decade there has been a paradigm shift in our understanding of the DNA mediated cell death mechanism as a result of radiation exposure. This has been based on a wealth of evidence, demonstrating that not only damage to cell nucleus but also cytoplasm and cellular membrane can lead to cell inactivation. It was also shown that cells, directly affected by radiation, can send signals, which cause biological responses in distant neighbouring cells. This phenomenon is currently known as the Radiation Induced Bystander Effect (RIBE), describing the processes occurring in cells not directly traversed by an ionising track, but which are affected by signals from irradiated cells [26]. RIBE embraces a variety of the experimentally observed non-targeted effects (including radiation induced adaptive responses[27, 28], low dose hypersensitivity [29], genomic instability [30] increased cell death and mutations [31-33] which are not necessarily detrimental, depending on how

these associated effects are considered from a radiation protection or radiation therapy point of view in normal or cancerous tissues.

2.3. Biological responses to ionising radiation.

2.3.1 Conventional understanding of the impact of ionizing radiation on living structures.

Ionising radiation is an effective damaging agent that affects DNA directly or interacts indirectly via chemical reactions with numerous radiolytic reactive products, such as $OH^{\cdot}$, $H^{\cdot}$, O_2^{-}, and H_2O_2. Both direct and indirect processes will cause different radiation induced DNA lesions such as: single strand breaks (SSB), double strand breaks (DSB), 20 types of base damage, DNA – DNA and DNA – protein cross-links, and multiple damage sites (MDS) [34]. Nearly two thirds of the radiation induced DNA lesions are caused by indirect action of sparsely ionising radiations such as γ or X rays with the remaining third causing the direct DNA damage[35].The amount of energy transferred to DNA and the type of lesions generated depend on the dose, dose rate and the nature of the ionising radiation (IR) - densely or sparsely. Table 2.1 below [36] compares biological end points created after delivery of 1Gy of radiation of different qualities – densely IR or high Linear Energy Transferred (LET) radiation such as α particles, protons or heavy ions; and sparsely IR or low LET radiation such as γ or X rays and electrons.

Radiation Impact	Low LET	High LET
Number of tracks in nucleus	1000	4
Number of ionization in nucleus	100000	100000
DNA SSBs	850	450

DNA DSBs	40	70
DNA protein cross links	150	-
Chromosome aberrations	1	3
Lethal lesions	0.5	2.6
Cells inactivation	30%	85%

Table 2.1. A comparison of various types of radiation induced damage generated by the same quantity but different quality of radiation exposures [36].

SSBs and base damage usually don't correlate with cell killing [34] and cannot be considered to be lethal. However DSBs, which lead to chromosomal changes and can cause a significant loss of genetic material during cell division, will contribute to cell kill or mutation.

Mammalian cells usually respond to DNA damage by activating two important cell functions – either DNA repair mechanisms or cell cycle regulation [37]. Repair mechanisms are usually quite active in most mammalian cells and are regulated by cell cycle checkpoints. In a short period of time, after DNA lesion recognition and assessment, a cell has to decide either to "commit suicide" (usually through apoptosis) or to repair damage, aiming first of all to preserve the reproducibility and integrity of genome, by following either of three main pathways:

I. Cells arrest in the cell cycle progression. Regulation of cells propagation through the cycle are governed by specific intracellular enzymes known as cycling dependant kinases (CDKs), which enzymatic activity "switch on" by the phases specific proteins (cyclins) and will allow cell transitions from one phase to another. All events during the cell cycle must follow a strict order to ensure that next event will not occur until the last event has been completed. These processes are regulated by a number of check point genes at three main positions in the cell cycle: G_1/S checkpoint, S-phase checkpoint and G_2/M check point. The genes sense the lesion, initiate

checkpoint signals and activate protein kinases, which will phosphorylate critical targets and result in cell cycle arrest.

II. Repair of DNA damage. After damage recognition and cell cycle arrest, a cell initiates an attempt to repair itself. For DSBs there are two main pathways – non-homologous ends joining (NHEJ) and homologous recombination (HR). NHEJ is initiated by DNA dependant protein kinas complex (DNA-PK), which localises broken ends and bind to them. HR takes advantage of the availability of the sister chromatid on the homologous chromosome and uses them as a template to repair damage.

III. Initiate apoptotic cell death. If a DNA lesion cannot be repaired the cell will "commit suicide" usually via apoptosis – the genetically regulated physiological process of removing damaged beyond repair cells.

Figure 2.1 is the simplified demonstration of the cellular responses to radiation induced damage.

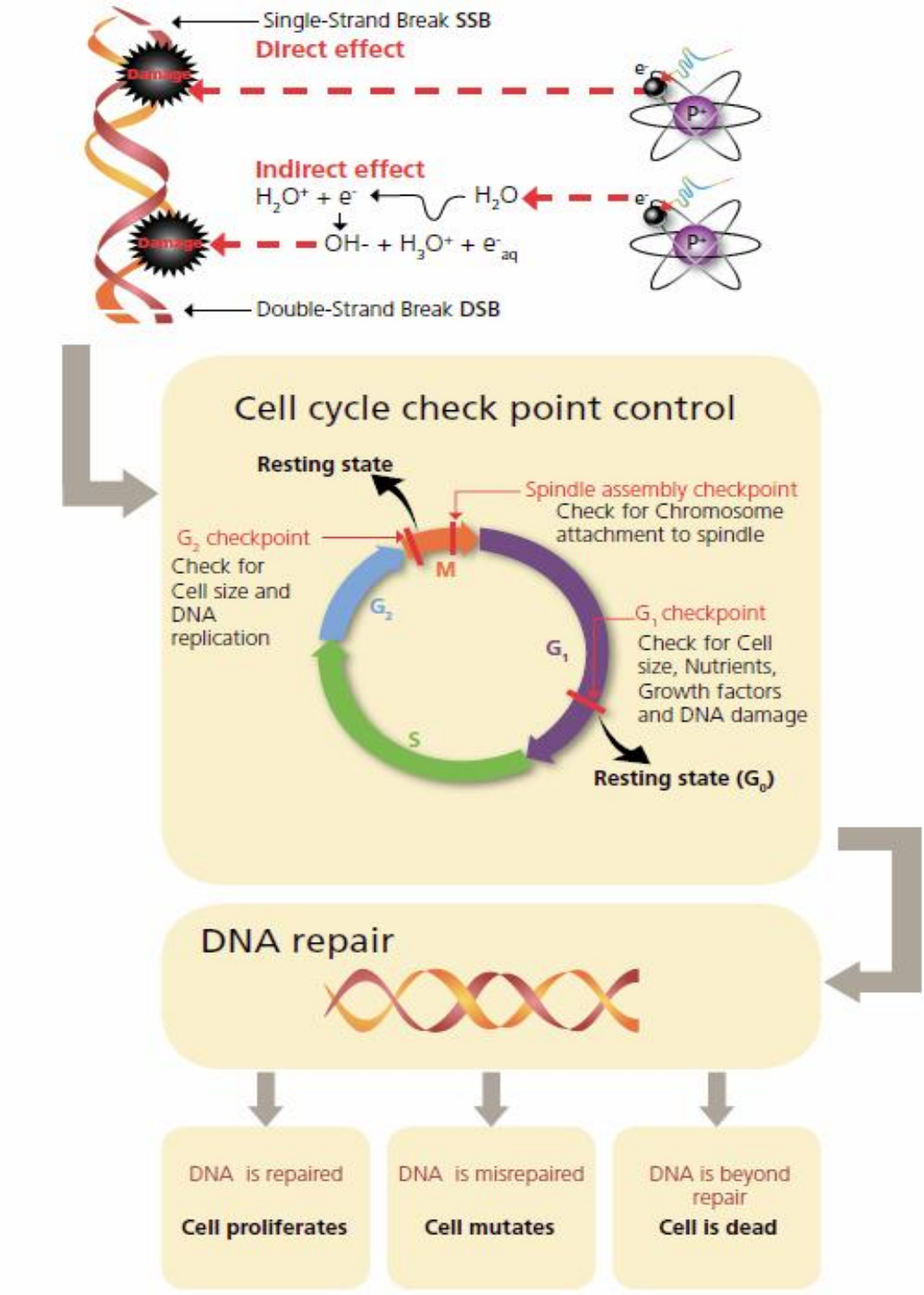


Figure 2.1. Schematic demonstration on how ionising radiation can affect cells.

Ionising radiation produces a variety of DNA damage and not all of them are lethal. Only around 1-2% of DSB are lethal, while most SSBs and DSBs can be successfully repaired [37].

2.3.2. Paradigm shift in radiation biology.

In the last decade the long-held DNA centric model has been questioned due to experimental evidence [22, 38-40], demonstrating biological responses in cells not directly affected by ionising radiation which cannot be explained with the DNA-direct-damage cell killing mechanism. These radiation-induced phenomena in non-targeted cells include increased radio resistance, adaptive responses, long-lasting alterations in gene expression, genomic instability, sister chromatid exchanges, low dose hyper- radio sensitivity, mutagenesis, reproductive cell death, micronucleus formation, increased apoptosis.

All these associated effects have been termed as *radiation-induced bystander effects* or *non-targeted effects*, and is sometimes referred to in the literature as a paradigm shift in target theory. Figure 2.2 demonstrates the comparison between conventional theory on cell killing process after radiation exposure and new paradigm in radiation biology.

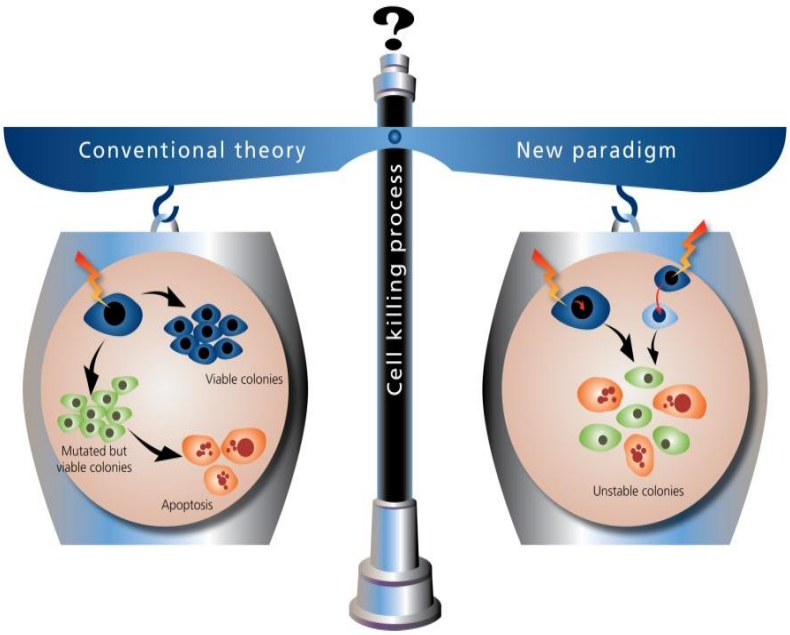


Figure 2.2 Schematic presentations of the cell killing pathways according to the conventional DNA-direct damage model and considering extra-intra-cellular signalling.

Applicability of either of these cell killing concepts to modern radiobiology is controversial and requires better understanding of the radiation induced bystander phenomena. In order to do so, cell communication must be considered and properly understood to allow interpretation of the underlying mechanisms associated with RIBEs.

2.4. Radiation induced responses observed in indirectly affected cells.

2.4.1 Possible mechanisms responsible for signal induction and propagation.

The ability of cells to communicate with one another plays a crucial role in the radiation induced bystander phenomenon. It has been known for many years that some cells (including bacteria, yeast and mammalian cells) can produce signals which affect other cells, govern basic cellular activities and coordinate cell actions by regulating cell growth, replication and death. Coordination of cell growth and differentiation are regulated by polypeptide molecules called growth factor or *cytokines*. Each cytokine, usually a small monomeric single chain polypeptide, but sometimes dimeric two chain amino acids, selectively bind to specific cell-surface receptor proteins in the process called transphosphorylation, which results in the transaction of two phosphoric groups from one compound to another. Signalling molecules at the cell surface communicate with cytoplasmic molecules and propagate signals through the cascade of different events which may affect DNA.

Another route for signal transduction involves reactive environmental agents, which can pass through the plasma membrane to the cytoplasm, due to their chemical compositions, and then bind to specific receptor molecules causing signal progression. Figure 2.3 demonstrates the possible pathways involved in bystander signalling.

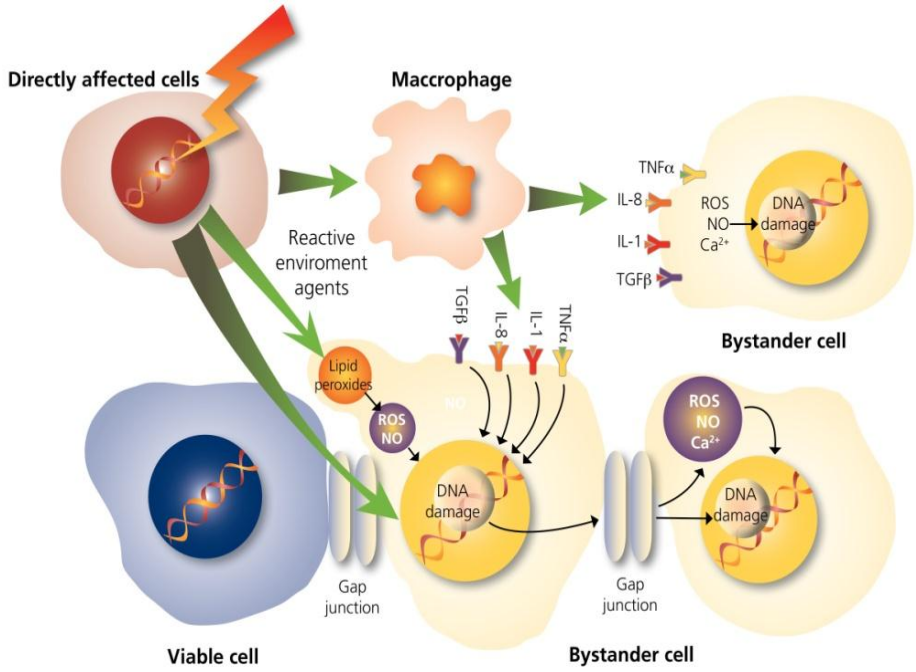


Figure 2.3 Schematic pathways of the possible pathways and key proteins involved bystander signalling.

Cell-to-cell communication is a complicated multistage process. However published literature [36] identified two main pathways of cell signalling involved in the radiation induced bystander phenomenon - short range Gap Junctional Intercellular Communication (GJIC) and long range Distant Cell Signalling Intercellular Communication (DSIC), mediated by soluble transmissible factors and propagated by Brownian active or passive diffusive motion.

Gap junction intercellular proteinaceous channels in cell membrane are very important dynamic structures which allow ions and small molecules to pass through from cell to cell and play a crucial role in cell growth and oncogenic transformation. Their important role in RIBE was postulated in many review articles, and was proven in some experimental works [41]. While the nature of the factors passing through gap junctions from the directly affected cells to the neighbouring cells or extra cellular environment has not yet been verified, some works suggested [42] that it had to be the protein-like molecules due to their abilities to survive one freeze/thaw cycle and the sensitivity to

heat [43] and, possibly, of epithelial origin [44]. Taking into account the size of the gap junction, there are molecules which **can** go through channels, such as water, ions, sugar, nucleotides, amino and fatty acids, small peptides, drugs and carcinogens. There are molecules which **cannot** go through gap junctions, such as heavy proteins, lipids and RNA. Nikjoo and Khvostunov in their modelling work [16] estimated that bystander signals in confluent solution can be diffused via GJIC by molecules which are less than 2 nm in diameter, assuming a spherical shape, and in the range of 1 up to 10 kDa molecular weight.

With regard to radiation exposure there are at least two main factors which play an important role in radiation induced bystander signalling, these being the quality and the quantity of the radiation. The involvement of the quality of radiation exposure in RIBE signalling pathways has been questioned and some experimental work [45] suggested that the gap junction intercellular communication is more likely to be induced by high LET radiation. Whereas bystander signal propagation, mediated via distant intercellular communication mechanisms, is more likely to be triggered by low LET radiation. Thus, Mothersill and Seymour [46] demonstrated that the cell to cell contact is not required to induce bystander responses in non-targeted cells after low LET irradiation.

The quantity of radiation exposure is another controversial point. In contrast to the direct radiation damage concept, where system response increases proportionally with increased dose, RIBE supposedly is only a low dose phenomenon. Some experimental work [31] suggested saturation of bystander responses at relatively small doses below 1 cGy, but which can be induced following radiation exposure levels as low as 5 mGy [47, 48] of low LET x-rays or 1 alpha high LET particle traversal [33]. The controversy of such a relationship can be explained by considering that high dose radiation causes more pronounced direct DNA damage; whereas at the low level of radiation exposure cellular signalling becomes more prominent and causes radiation induced bystander responses in non-targeted cells.

Cellular signalling is the key factor which plays an important role in understanding the concept of the radiation induced bystander phenomenon. Many possible mechnisms of cellular signalling from directly irradiated cells (or extra cellular compartments) to non-irradiated cells can be hypothesised. Table 2.2 is the brief summary of the possible mechanisms involved in initiating and mediating RIBEs.

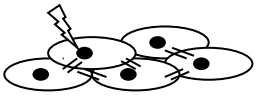
a	Cell directly affected by radiation sends signals to non-irradiated neighbouring cells via gap junctions. 
b	An irradiated cell produces soluble proteins, such as cytokines or growth factors, which can induce responses in untreated cells by activating surface receptors in them, which recognise the signalling proteins. 
c	Extra cellular matrix and fluids directly affected by radiation can create reactive oxygen species and reactive oxides of nitrogen, or trigger the release of matrix-bound soluble proteins, which can affect neighbouring cells. 
d	Directly irradiated cells or extra cellular fluids produce transmissible factors, which result in release of reactive oxygen species, interactions of which with the extra cellular matrix may trigger release of other factors that can affect both directly irradiated and bystander cells.

Table 2.2 Possible mechanisms of cellular signalling involved in RIBEs.

In spite of the unclear origin of the radiation induced bystander signals some of the mechanisms and key pathways at the molecular level have been identified [49]. Among them are CycloOXygenase 2 (COX2), Death Receptor 5 (DR5), InterLeukin (IL), Jun N terminal Kinase

(JNK), Nitric Oxide (NO) and NO synthase 2 (NO2), Reactive Oxygen Species (ROS), Transforming Growth Factor β (TGβ) and its receptor (TGβR), Tumour Necrosis Factor α (TNF α).

2.4.2. Biological endpoints observed in non-irradiated bystander cells.

RIBEs embrace the variety of the biological end points observed in cells not directly hit by ionising radiation. These end points include sister chromatid exchange, micronuclei formation, cell killing/delayed cell death, increased intracellular level of ROS/NO, apoptosis, gene and protein expression changes, radio adaptation, chromosomal instability, neoplastic transformation, increased level of calcium fluxes and histone H2AX phosphorylation. All these responses can be either detrimental or beneficial for cells and can lead to:

- Additional cell killing
- Cell mutation
- Radio-adaptation.

Radiation induced bystander killing and radiation induced adaptive responses are two conflicting, but probably related phenomena. Zhou *et al*, [50, 51] demonstrated increased mutagenesis in non-irradiated cells, which were in direct contact with cells whose nuclei were traversed by alpha particles. However, a significant decrease in mutagenic responses was observed after cells were pre-treated with a low dose (1 cGy) of 250 kVp X-rays.

Sawant S.G. *et al* [52] reported an increased magnitude of bystander killing in their experiment with increased number of alpha particles per cell. However, cell pre-treatment with 2 cGy of 250 kVp X-rays six hours prior to irradiation “cancelled out about half of the bystander effect, produced by alpha particles”. Figure 2.4 demonstrates results obtained from this experiment.

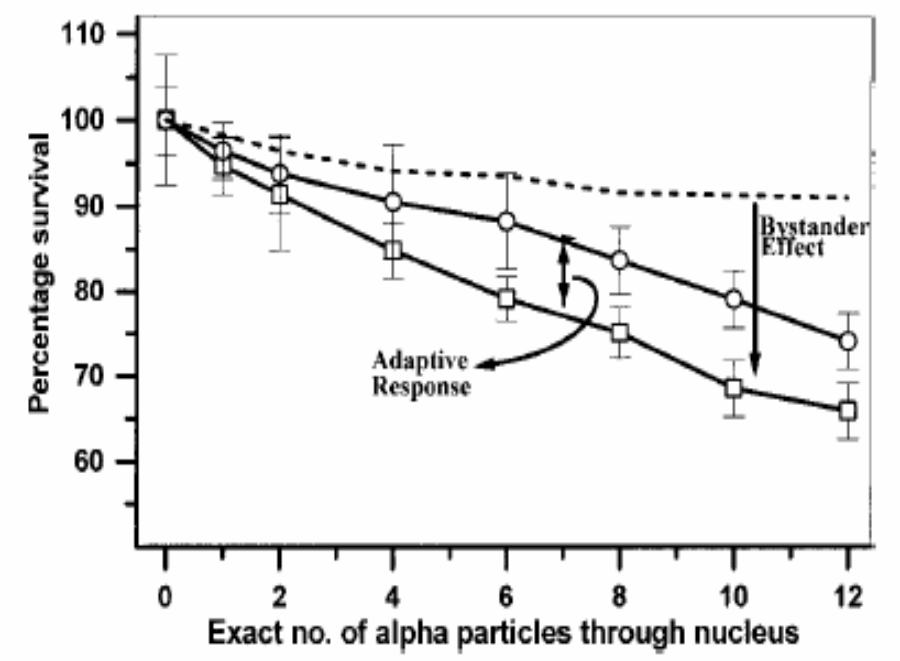


Figure 2.4. Experimental results obtained from Sawant et al compare adaptive vs bystander responses in C3H10T1/2 cells. The dotted line represents the percentage of cells which **would be affected** when 10% of cells are exposed to the certain amount of alpha particles. This is in contrast to the square line demonstrating the cell survivals which **were affected**. And the circle line represents adaptive responses in the cell survival which were irradiated with the same amount of alpha particles but pre-treated with low 2cGy x-rays 6 hours before micro-beam irradiation.

Apart from the extensive experimental work performed in the past to detect non-targeted effects of ionising radiation, a significant amount of research has also been done to quantify some dynamic and kinematic characteristics of this phenomenon, such as time dependence and spatial distribution. However, observed data revealed difficulties in its interpretation and determination of the magnitude of RIBEs due to differences in experimental conditions such as experimental design, end points scored, cell culture morphology and geometry – either 2D cell monolayer or 3D tissue, i*n vivo* or *in vitro*, quality, quantity and the methods of radiation delivery.

Scheittino *et al* [53] for example observed cells in sparsely populated culture, which failed to form colonies **3 mm** away from cells directly irradiated with ultra soft 278eV X-rays.

Hu *et al* [54] reported γ-H2AX foci, which indicates DSBs formation, **7.5 mm** away from densely populated cells irradiated with 3.8 MeV α particles. Time dependence was also analysed in this experiment and it was stated that "positive cells with DSBs", but not targeted by α particles, could be observed 2 min after irradiation reaching its maximum in 30 min.

Belyakov *et al* [55] in their *in vitro* experiment with 3D artificial epidermal human skin tissue systems observed micro nucleus formation **0.6 mm** and apoptosis **1 mm** away from cells directly irradiated with fixed number of α particles (7.8 MeV).

A couple of years later Sedelnikova *et al* [56] performed micro beam irradiation with 7 MeV He^4 ions of 3D human tissue models and observed formation of γ-H2AX foci **1 mm** away from the irradiated plane. She also confirmed the maximum of DSBs formation 30 min after irradiation.

Koturbash *et al* [57] found DNA damage, such as DSBs and increased level of Rad51, "**more than a centimetre away**" from tissues which were irradiated directly with 90 kV soft X rays in their *in vivo* experiment with nude mice.

2.5. Experimental approaches used to investigate RIBE.

Significant variation in the experimental designs and techniques used to investigate RIBE create difficulties in the data interpretation and analysis. However results can be systematised assuming that all experimental solutions used must include two parts - biological and irradiation set ups.

Biological set ups include:

1. Biological systems used to investigate RIBE such as:

- *In vitro* 2D confluent cells monolayer the technique used in the majority of early experimental work.
- *In vitro* 3D artificial human tissue model [58], which has morphological and growth characteristics of *in vivo* like system.

- *Ex vivo* human tissue samples [59]
- *In vivo* nude immune deficient mice model [60]
- *In vivo* partial animal body irradiation [61]

2. End points scored, which were listed previously, significantly vary in different types of assays used, depending on the biological system responses to be investigated.

Irradiation techniques used to investigate RIBE include:

1. External beam irradiation such as:

- X-ray broad beam irradiation with high energy linear accelerators or radiation emitting devices (such as Co-60 or Cs- 137 units). This technique was mainly used in so-called irradiated cells conditioned media transfer experiments in which non-irradiated cells are exposed to the media (cell growth solution), collected from the irradiated cells. It was postulated and demonstrated [42] that the intensity of the bystander signals depend on the dilution of the media conditioned by irradiated cells.
- X-rays broad beam irradiation with high energy (MeV region) linear accelerators and partially shielded irradiation field – technique which delivers inhomogeneous dose distribution to the *in vitro*[62] or *in vivo* systems.
- Charged particle micro beam irradiation. This relatively novel irradiation technique, that lead to RIBE discovery [63], allows the delivery of precise amounts of ionising radiation (such as the desired number of α particles) to each individual cell and its compartment, such as cytoplasm or cellular membrane [64] and also to its cellular environment, such as extra cellular matrix and fluids.
- Irradiation with low energy (kV region) external beam superficial x-ray radiation devices.

2. Internal irradiation with radioisotopes such as:

- Co-cultured radio labelled and unlabelled cells systems [60].

- Irradiation with short range β particles or Auger electrons emitted from isotopes.
- Irradiation with low fluencies of α particles [65].

Depending on the access to experimental apparatus and materials, researchers have developed and applied different techniques to investigate RIBE. A detailed discussion and analysis of all experimental techniques used to investigate RIBE is out with the scope of this paper.

2.6. Overview of the currently proposed mathematical models.

The radiation induced bystander phenomenon changes our conventional understanding of the target size and the associated biological responses to radiation, which is currently presented in radiobiology by the linear quadratic formalism. Thus changes in assumed target size might result in the necessity to revise currently used models. Unlike early studies on RIBEs, there is now moderate amount of data contributing to further understanding and quantification of this phenomenon, which has a potential to modify currently used radiation damage models.

Radiobiological models, similar to any other mathematically based models, fall into two main categories - theoretical or empirical. The theoretical models are based on the proven evidence of the processes involved and on the strong logical theory behind it. This is a time, resources and data consuming process, requiring significant amount of experimental work to be performed. On the other hand empirical models are derived to fit a particular experiment and are valid only for the given experimental conditions. The last method is broadly used for testing new phenomena and theories, including RIBEs. Table 2.3 provides short summary of the modelling approaches applied to the radiation induced bystander phenomenon.

Authors & Names	***Modelling Approach***	***Results & Conclusions Drawn***
BaD ***B****ystander* ***a****nd* ***D****irect damage,* ***Brenner et al [66]***	*Semi-empirical model, based on the data from micro beam experiment [67]. Binary approach - all or nothing. No distant signalling mechanisms were hypothesized, and therefore, such a model can only be applied in a situation when cells are in direct contact with each other. Two main parameters were analysed SF, survivals fraction, and TF, fraction of oncogenic transformation.*	*Bystander effects are important only at small doses – below 0.2 Gy and can be induced only in the small radiosensitive subpopulation of cell. The radio sensitivity of this subpopulation is due to the geometrical location rather than biological status. BS killing in the case of broad beam is negligible.*
BSDM (bystander diffusion model) ***Nikijoo and Kvostunov [68]***	*Semi-empirical model based on experimental works [67], [69] and Monte Carlo simulation. Bystander signals in confluent solution can be defused, by Brownian motion via GJIC channels by protein like molecules, which are less than 2 nm in diameter (assuming spherical shape) in the range of 10 kDa molecular weight and such signals are, normally, generated when cells are coming into a state of mitosis/apoptosis/necrosis. The signalling protein can react with the "targeted" cell when the distance between them less than half cell diameter. Two main parameters were analysed – cell survival fraction and cell transformation fraction.*	*BSDM confirmed the size and the origin of signalling molecules and demonstrated "that the bystander effect cannot be interpreted solely as a low dose effect phenomenon" and some responses can be observed at high doses as well as low doses.*
Little et al [70]	*Semi-empirical stochastic model based on the 17 parameters predicting cumulative probability of cells to be affected. Set of these parameters was based on the "most biologically plausible behaviour". This model analyses spatial location, cell killing and repopulation.*	*Not linear relationship with dose – saturation of the effect after 0.2 Gy.* *This model analysed unimportance of RIBEs after fractionation, which was demonstrated in some experiments [71].*
Microdosimetric model ***Stewart et al [72]***	*Stochastic model based on the microdosimetric principals derived from ICRU (report 36, 1983) to simulate emission signals and processing of cells death due to dose absorbed. Four main parameters were analysed: "number of signals generated by radiation-damaged cells, the number of sender (hit) and receiver (non-hitor hypersensitive) cells,*	*Signal production is either exponential or bi-exponential function of absorbed dose.* *Exponential model predicts the saturation of medium-borne signals after a cell is hit at least once.* *Bi exponential model*

	the probability that a signal is transmitted to and detected by an undamaged cell, and the probability that a cell exhibits a specific response after."	*demonstrated saturation of signals in 5 to 100 mGy dose region.* *Liu et al [73], based on the Stewart microdosimetric model and data for HPV-G cell line, could determine the effective diameter of the target, which emits signals after irradiation – 2 μm.*
State vector model (SVM) ***Schollnberger and Eckl [74]***	*Semi-empirical multistage model, which incorporates cellular responses to IR such as DNA damage and repair, intercellular communication, both spontaneous and radiation-induced cell death and cell division. This model assumes that cells are being distributed within 6 different compartments, described by differential equations, and a "state vector denotes the amount of cells in each state at time t." Neoplastic transformation and radiation induced apoptosis were analysed in this model.*	*A protective apoptosis-mediated bystander effect (PAM) was found to be important at the doses before 200 mGy of low LET radiation. The time span indicating probability of PAM activation was determined to be 5 days.*

Table 2.3. Brief review on the modelling work of RIBEs

The main purpose of the majority of the modelling works listed above was to distinguish between the direct and indirect (bystander) components of radiation induced damage. Thus the very first Bystander and Direct damage model (which was applied to the Sawant's [75] micro beam-experimental data and where 1 in 10 cells were exposed to a certain amount of alpha particles) analysed surviving fraction (*SF)* and oncogenic transformation frequencies *(TF)* and expressed them as follows:

$$SF = 0.1q^N + 0.9F(N) \quad (2.1)$$

$$TF = (0.1\nu Nq^N + 0.9\sigma F(N))/SF \quad (2.2)$$

Where q is the probability of a cell to survive a single alpha particle traversal, N is the number of such a traversals, $F(N)$ is the fraction bystander cells which survive those traversals, v is the slope of the dose-response curve related to the linear direct component and σ is the fraction of bystander cells which are sensitive to oncogenic transformation.

The next model of the bystander phenomenon, ByStander Diffusion Model, applied the same micro-beam experimental data and analysed same *SF* and *TF* parameters which were expressed as follows:

$$SF = 0.1a^N + 0.9(1 - B_S) \quad (2.3)$$

$$TF = (0.1bNa^N + 0.9(1 - B_S)cB_C) / SF \quad (2.4)$$

Where a is the probability for a cell to survive a single alpha particle traversal, N is the number of alpha particles, B_S is the fraction of inactivated bystander cells, $B_C = B_S * f$ (where f is the fraction of non-hit cells), b and c are constant parameters obtained from micro-beam experiment [76].

The number of bystander signals (μ) which can be released by a directly inactivated cell was analysed in BSDM model as well. It was estimated as $\mu=8$ for high LET and $\mu=1.2$ for low LET radiation exposures.

The listed models don't represent all the modelling work performed to investigate RIBEs but can be considered to be the most systematic approaches to quantify and to predict behavioural patterns of still not fully understood phenomenon. In spite of different results and conclusions drawn from these models, some similarities can be identified in the methodological approaches and results obtained. However, these models have limited application and the numbers revealed are of limited values considering that the results are valid only to one (or a few) specific data set. More systematic

approach to all available data needs to be applied in RIBE modelling, which can be significantly improved by including clinical data whether it's possible.

2.7. Potential impact of RIBE in radiotherapy.

2.7.1. Key factors potentially relevant to modulating radiation induced bystander responses in radiotherapy.

There are well known radiobiological factors referred to as the 5 "R"s of radiation biology, which are crucial for the efficacy of any radiation therapy treatment and can be relevant to modulate radiation induced bystander mechanisms in vivo. Among them are:

- **R**e oxygenation.
- **R**epopulation.
- **R**edistribution along the cell cycle.
- **R**epair.
- **R**adio sensitivity.

2.7.1.1. Radiation induced bystander responses under hypoxic condition

Published literature is not clear about RIBEs under hypoxic condition. However there are strong reasons to expect radiation induced bystander responses to be more pronounced in well oxygenated healthy cells compared to angio-vascular-defective tumour cells with poor oxidative metabolic activity. An oxidative stress in such cells can lead to decreased or even absent cytotoxic bystander components mediated by increased intracellular levels of reactive oxygen and nitric oxide species.

Considering this, there is a hypothetical possibility to improve treatment therapeutic outcome once we are able to modulate bystander responses in healthy and cancerous cells separately.

2.7.1.2. Radiation induced bystander responses and repair mechanism.

There have been some discussions on the protective role of RIBE involved in cellular repair mechanisms. It was hypothesised that some of radiation induced bystander responses in non-irradiated cells can trigger termination of cell division in DNA-damaged or improperly-repaired cells. Considering this, it is logical to expect significantly larger bystander deaths in the repair-deficient cells. Thus Mothersill e*t al* [77] tested this hypothesis in several repair-deficient cell lines and observed "moderate to severe bystander induced death effects" in those cell lines and their surviving progeny compared to normal repair-proficient lines, which had "very much less severe or absent bystander-inducible effects on cloning efficiency". Another study [78] revealed that the differential nature of DNA damage response mechanisms in directly targeted and bystander cells in theory has a potential to improve radiotherapy treatment outcomes [76] once therapeutic agents can be differentially modulated as targeted and non-targeted effects of radiation.

2.7.1.3. Radiation induced bystander responses and cell cycle position.

The cell cycle position during irradiation plays a very important role in the efficacy of the treatment. For example, the fact that cycling tumour cells are more radio sensitive allows us to improve the therapeutic outcome by blocking tumour cells in the radiosensitive phase with radio-sensitisers and killing with radiation. Unfortunately, very little is known about the abilities of cells to experience radiation induced bystander responses, depending on the cell cycle position. However it

is reasonable to expect that some of the radiation induced bystander responses might be caused by the failure in cell cycle check-points after radiation exposure. It was presumed that the G2 phase could be a very possible candidate that may be involved in bystander factor production and responses [79]. Thus experimental work performed by Short *et al* [80] supported this presumption by demonstrating the involvement of the G2 cell cycle check point in the phenomenon called low dose hyper-radio-sensitivity, which represents a deviation from linear-quadratic dose response relationship at low (below 10 cGy) doses. Joiner *at el* [81] investigated this phenomenon and suggested that at low doses (lower than some "putative threshold") cells lose the ability to sense damage or at least to recognize it quickly. Consequently, such a cell progresses through the cell cycle un-repaired or repaired inefficiently. Another study performed by Belyakov *et al* [82] confirmed the importance of the cell proliferation status (which is directly correlated to cell cycle position) in the magnitude of radiation induced damage in bystander cells.

2.7.1.4. Radiation induced bystander responses in fractionated radiotherapy modalities.

The purpose of fractionation in radiotherapy treatment is to deliver a sufficient dose to kill the tumour but at the same time allowing healthy normal tissues to recover. Very little is known about radiation induced bystander responses in fractionated radiotherapy regimes because the majority of experimental work performed used single dose, high dose rate radiation exposure. Few studies have been done to research this field. A group from Canada [83] looked at the dose rate effect on radiation induced bystander responses and reported differences in the bystander survival after changing the dose rate. Mothersill *et al* [47] investigated the impact of fractionation on RIBEs. They observed more toxic effects of multiple radiation exposure in bystander cells compared to just a single fraction and suggested that bystander factors produced "in vivo" may reduce the sparing effect of the dose fractionation. On the other hand, it is logical to expect that multiple radiation induced bystander

responses (such as additional cell mutation or killing) in cancerous cells can improve the efficacy of treatment after a fractionated radiotherapy regimen. Thus, it is reasonable to assume that bystander oncogenic cells, which still belong to the same tumour and are located beyond the outlined planning treatment volume (in micro-spread of the disease), will respond to each fraction as to a single unique dose and result in additional killing after receiving signals from directly irradiated cells.

2.7.2. Potential detrimental and beneficial effects of radiation induced bystander responses in radiotherapy.

Normal cells and tumour cells differ significantly in their physiology in many ways, such as immortality of tumour cells (due to activation of their own enzyme telomerase); more rapid growth kinetics; abnormalities in cell-to-cell communication due to defective gap junction channels; defective vacuolisation and poor oxygenation. Therefore, it is reasonable to expect significant variation in bystander signalling induction and propagation between normal and cancerous cells.

Potential impacts of RIBEs in the clinical environment, such as radiation therapy, can be either *detrimental* or *beneficial* depending on their possible roles. Table 2.4 briefly discusses the possible advantages and disadvantages of this phenomenon in radiotherapy.

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	Normal Cells	**Cancerous Cells**
Radiation induced killing in bystander cells	Neither – cannot be harmful as millions of cells die in a human body each day. However *significantly* increased killing of normal adjacent cells can contribute to late acute toxicity and therefore has a potential to	**Beneficial** – potentially can contribute to the additional killing of tumour cells and elimination of adjacent pre-malignant cells [84]

	be **detrimental.**	
Radiation induced mutation in bystander cells	**Detrimental** – possibility to cause late organ damage and radiation induced secondary cancer.	**Beneficial** – potentially can contribute oncogenic transformation and differentiation of undifferentiated tumour cells.
Radiation induced radio adaptation in bystander cells	**Beneficial** – can potentially protect healthy tissues (especially important during fractionation).	**Detrimental** – possibility to contribute toward oncogenic survival and tumour recurrence.

Table 2.4. Possible advantages and disadvantages of RIBEs.

There is some evidence to support the hypothesis that *in vivo* RIBE may be of relevance to human health. Abscopal effects of ionising radiation, which were defined in early literature "as radiation responses in tissues that are widely separated from an irradiated area" were described nearly half a century ago by Nobler [85] in patients with chronic leukaemia and by Parsons *et al* [86], who also reported "changes in sternal marrow following roentgen-ray therapy" administered to the spleen of paediatric patients with chronic granulocytic leukaemia. Hollowell and Littlefeild [87] reported abscopal effects (lymphatic damaged chromosomes) in the untreated cells after they were kept in the plasma obtained from patients irradiated with X-ray. A year later Scott [88] confirmed these findings by observing chromosomal aberration in the lymphocytes exposed to the plasma of irradiated patients.

There are many works [89], [90], [91] that have suggested an increased possibility of a second primary cancer after radiotherapy treatment. These findings have not proved that radiation (but not any other agents and life style choices) causes induction of second malignancies. However, recent data on radiation induced genomic instabilities and mutation in non-targeted cells make it reasonable

to hypothesise that radiation induced bystander responses could be involved in multistage processes leading to carcinogenesis.

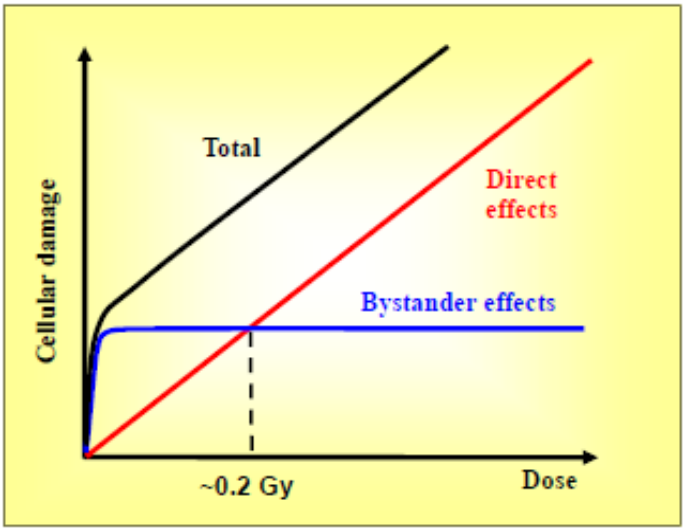

Figure 2.5. Schematic diagram obtained from Oleg Belyakov, is based on the majority of the experimental work performed to investigate RIBEs, and demonstrates contribution of bystander and direct components of radiation induced damage.

In the order to evaluate the possible impact of the RIBE in radiotherapy by reconsidering the real biological target size versus presumed radiation boundaries one needs to examine this phenomenon *in vivo.*

RIBEs *in vivo* can only to be investigated when less than whole body is irradiated and the information, such as dose and dose volume distribution, can be obtained. Therefore the studies on atomic bomb exposure and accidental exposure survivors as well as information on second malignancies after total body irradiation (TBI) cannot contribute to RIBE investigation *in vivo.* The most informative data in this regard can be based on the studies of the second primary cancer rates

after radiotherapy in children or/and young adults with high survival rates and long follow up potential.

2.7.3. Potential implication of RIBEs for radiation-based therapies.

The radiation induced bystander phenomenon is potentially relevant to any radiation-based treatment modality in terms of its affect on therapeutic outcomes.

For example, *targeted radionuclide therapies* in conjunction with *gene therapies* have a very good chance of selectively modulating radiation induced bystander responses. Boyd et al [92] compared bystander responses induced by external beam irradiation to radiopharmaceutical induced bystander killing. It was found that in contrast to external beam irradiation, where an increased degree of bystander killing was observed only at low doses and then saturated at higher doses, treatment with radio-nuclides did not reveal low-dose-saturation in bystander responses. These studies incorporated both low and high linear energy transfer emitters and suggested LET dependence on the degree of radio-pharmaceutically induced bystander responses. Later this assumption was supported by another experimental work [93] from Massachusetts University by Mairs *et al.*

Brachytherapy is another treatment modality where RIBEs can potentially affect treatment outcomes. Thus, Chen et al [94] demonstrated significantly increased clonogenic death in non-targeted adjacent cells after exposure to low dose rate I-125 seeds, a radioisotope widely used in clinics for prostate brachytherapy.

Recent developments in the delivery of *external beam* radiation therapy including intensity modulated radiotherapy (IMRT), helical tomotherapy and heavy particle ion therapy indicate the necessities for further investigation of RIBEs that potentially can contribute either to effectiveness of

treatment or to increased radiation risk. There is some probability that non-targeted effects of ionising radiation can modulate therapeutic outcome under conditions where significant variation in dose steepness gradient, dose rate and increased irradiation delivery time are present. Thus Bromley et al [95] investigated an effect of the variation in the dose steepness gradients on the cells survival and found it to be different depending on the width of the penumbra of an x-ray radiation field. Other studies [96, 97] investigated dose-rate effects and observed variation in cell survival, which was correlated by variation in the external beam dose delivery scheme such as continuous arc IMTR, prolonged helical tomotherapy and segmented seven fields irradiation.

2.7.4. Can treatment outcomes to be improved by introducing RIBEs to clinicians?

All radiotherapy teams (including radiation oncologists, medical physicist and radiotherapists) have an opportunity to undertake research and contribute towards the new understanding of radiation induced damages and associated effects. It is especially relevant to radiation oncologists who have unique opportunities to follow up patients and make assessments before, during and after radiotherapy treatment.

Most of the experimental work on RIBEs was performed in artificial conditions, which is logical when it comes to investigating new theories. However, more clinical research on non-targeted effects after radiation based therapies needs to be undertaken. Such studies may consist of methodically collected patient data (including treatment conditions, prescribed drugs, early/late toxicity) and methodically collected/tested patient samples (biopsies and normal tissues) before, during and after radiotherapy. It is not an easy task to design and perform such a long term epidemiological study, however raising clinicians' awareness of the existence of the radiation induced bystander

phenomenon and its possible relevance to clinical outcomes after radiotherapy treatment may contribute to further research.

2.8. Conclusions and further work.

In the last decade radiobiological science has undergone major development by shifting from the DNA centric model to a broader understanding of the radiation induced damage. Extensive in vitro studies revealed few answers with regards to the radiation induced bystander phenomenon by determining the ways of signaling and naming some key factors involved in signal propagation. A small number of modeling works, which was mainly based on *in vitro* studies, was performed to analyze the behavioral pattern and to quantify this phenomenon. Radiation induced bystander responses with respect to radiation therapy can be classified as being detrimental (risk of second cancer development) or can be viewed as a beneficial factors (additional killing in tumor population or radio adaptation). Considering the importance of this phenomenon in radiotherapy, more *in vivo* data, preferably even clinical patient data, needs to be obtained. The impact of RIBEs in application to novel radiotherapy treatment techniques such as intensity modulated radiation therapy and tomotherapy, which deliver highly conformal doses to tumors, but cover bigger volumes at the low dose region where bystander responses are more pronounced, needs further investigation. This paradigm shift in the target theory is significant from a radioprotection point of view and it also has the potential to reassess radiation damage models currently used in radiotherapy for Tumour Control Probability (TCP) and Normal Tissue Complication Probability (NTCP) evaluations. The efficacy of the radiotherapy treatment techniques can be improved by developing new strategies to modulate beneficial and detrimental bystander responses in tumor and normal tissues.

Chapter 3.

Experimental Investigation of the Cell Survival in Dose Cold Spot in Communicating and Non-Communicating Cells'.

A version of this chapter with content formatted for publication has been submitted as: *Sjostedt, S., Bezak, E. and Marcu, L. (2012) 'Experimental Investigation of the Cell Survival in Dose Cold Spot in Communicating and Non-Communicating Cells'.*

3.1. Abstract.

The aim of this work was to investigate the impact of intercellular contact during radiation exposure on cell survival in regions of reduced dose. The PC3 human prostate adenocarcinoma cell line growing in the laboratory conditions was irradiated to assess clonogenic and apoptotic cell deaths with the ultimate goal to investigate cell survival in a dose cold spot. Radiation induced cellular damage in a 20% lower dose region, compared to the cell survival receiving 100% of the prescribed dose (2 Gy), was assessed for the experimental set-ups when under-irradiated shielded cells were either in direct contact with cells receiving 2 Gy or irradiated separately.A significant ($P<0.001$) decrease in cell survival was found when treated in the same flask cells received either 100% or 80 % of the prescribed dose versus non-irradiated cells. However, in the experimental conditions when cells received either 100% or 80% of the prescribed dose but were treated separately, the mean difference in cell survival between non-irradiated control and under irradiated by 20% cells was not significant ($P>0.05$). This was contrary to the significant decrease ($P<0.001$) in

cell survival receiving 100% of the planned dose versus control group. Additionally, significant reduction in cell survival was observed in cells which were under irradiated by 20 % and located in the same flask with cells receiving 100% of the prescribed dose compared to cells treated with 80% of the prescribed dose but irradiated separately from cells received full dose. For the given cell line under existing growing and treatment conditions the cell survival in the dose cold spot region was significantly lower when under-irradiated cells were in contact with the cells receiving 100% of the prescribed dose compared to the cellular survival obtained from the under-dosed cells, by the same amount of radiation, which were treated separately.

3.2. Introduction.

Prostate cancer is the most common cancer diagnosed in Australia and the second highest cause of cancer deaths in men according to the Australian Institute of Health and Welfare [98]. Radiation therapy combined with hormone therapy has been quite successful in curing this disease [99] and it represents one of the most common treatment options for men with intermediate-risk, low staged (T1 and T2) disease. There are several treatment modalities currently used worldwide to deliver radiation to the prostate gland, which include: permanent trans-perineal low dose rate brachytherapy using radioactive seeds, external-beam radiotherapy combined with low or high dose rate brachytherapy boost, intensity-modulated radiotherapy (IMRT) and 3D conformal radiotherapy. Treatment success of these modalities is usually assessed by achieving local tumour control and avoiding normal tissue complications. The outcome of radiation treatment relies, in part, on the design of the treatment plan. In spite of the considerable advances in the novel treatment planning algorithms, the radiation dose may not always be delivered as desired. This is the result of limitations in image acquisition, anatomy definition, treatment plan optimization and complex anatomical configuration of treated and

healthy structures [100]. Thus, the limitation of the majority of imaging devices to visualize a tumour does not allow identifying the extent of spread of sub-clinical disease. The subjective nature of treatment planning, in particular in outlining target volume and organs at risk, results in the uncertainties arising from inter- and intra-observer variations in target volume delineation [101]. Geometric uncertainties associated with limitation of imaging devices, expected variations of target volume definition, inaccuracies or variations in treatment set-up and delivery can be resolved by applying appropriate margins [102] [103] and by implementing image guided radiation therapy on a daily basis.

However, optimal dose distribution can be still difficult to achieve due to the complex anatomical configuration of targeted organs and healthy radiosensitive tissues laying in close vicinity to the high radiation dose gradient. For example in the case of a high dose rate brachytherapy boost to the prostate gland, a radiosensitive urethra (which is partly laying within a prostate) cannot be irradiated above the certain limits - usually 90% and higher of the prescribed dose [104]. Similarly there are also dose limits for parts of the bladder and radiosensitive rectum adjacent to prostate gland, depending on clinically implemented treatment protocols. The conflicting demands of a desired radiation dose to tumour volume and dose limits to healthy structures may result in generation of dose cold spots in a treatment plan – a well-known problem for radiotherapy planners. Theoretically, dose cold spots in a small area of the treated volume should not have a significant effect on the objective value of a physical dose plan, especially taking into account that only 1% or less of colonogenic cells are capable of unlimited proliferation within a tumour [105]. However, it has been shown that tumour control probability (TCP) can be greatly diminished as a result of dose cold spot. For example, Tome *et al* evaluated TCP loss due to dose cold spots within a tumour as a function of the dose reduction and the under-dosed volume. This study demonstrated that dose cold spot can be deleterious to TCP if the cold dose is lower by more than 10% of the prescribed dose for

fractions of cold volumes as small as 1% [106]. This indicates that cold spots cannot be ignored and require time-consuming adjustments to a treatment plan.

Recent discoveries in the areas of interaction processes between ionising radiation and living structures (which include radiation-induced adaptive responses, increased cell death and mutations, low dose hypersensitivity and genomic instability observed in non-targeted by radiation cells [107] [108]) change our understanding of the DNA-centric cell killing model and broaden the currently expected target size for ionising radiation. These phenomena are known in literature as a Radiation Induced Bystander Effect (RIBE) which was defined as the "ability of cells affected by an agent to convey manifestation of damage to other cells not directly affected by an agent"[109]. The increased target size may have important clinical implications for health risks associated with radiation exposure. The multifaceted nature of this phenomenon such as additional killing, mutation or radio adaptation in normal or cancerous cells results in controversy in terms of being either potentially detrimental or potentially beneficial to radiotherapy treatment outcome.

In this study we investigated an impact of cellular communication on cell survival in dose cold spots. Cellular signalling is the key factor which plays an important role in understanding the concept of the radiation induced bystander phenomenon. Many possible mechanisms of cellular signalling from directly irradiated cells (or extra cellular compartments) to non-irradiated cells can be assumed [110]. Even though the precise mechanism is unknown, there is substantial evidence that bystander signals may be transmitted by direct gap junction communication and by media soluble factors [21]. Experimental work [45] suggested that the gap junction intercellular communication is more likely to be induced by high LET radiation. Whereas bystander signal propagation, mediated via distant intercellular communication mechanisms, is more likely to be triggered by low LET radiation [111].

The quantity of radiation exposure is another controversial point. In contrast to the direct radiation damage RIBE supposedly is only a low dose phenomenon, although this presumption is

still supported by the limited number of studies [112]. Studies [113] suggest a saturation of the bystander response at doses below lcGy. The induction of a bystander response can, however, occur at radiation doses as low as 5 mGy [48] for low LET x-rays or at one high LET alpha particle traversal [33]. 'The controversy of such a non-linear dose relationship can be explained by considering the high levels of direct DNA damage at high doses of radiation as the dominant mechanism of cell kill. Whereas at the low level of radiation exposure cellular signalling is more prominent.

The main objective of this work was to investigate whether there are any differences in cell survival in under-dosed regions in freely communicating cells versus non-communicating cells. In the current study the authors did not aim to differentiate between the scenarios of inter-intra-cellular communication and assumed all mechanisms were involved in any observed RISE. The main intention of this work was to determine whether RIBE lead to reduced cell survival in region of a cold dose spot for a dose range relevant to radiation therapy.

3.3. Experimental methodology.

The plausible working assumption and the main structure of the experimental approaches are briefly outlined in Figure 3.1.

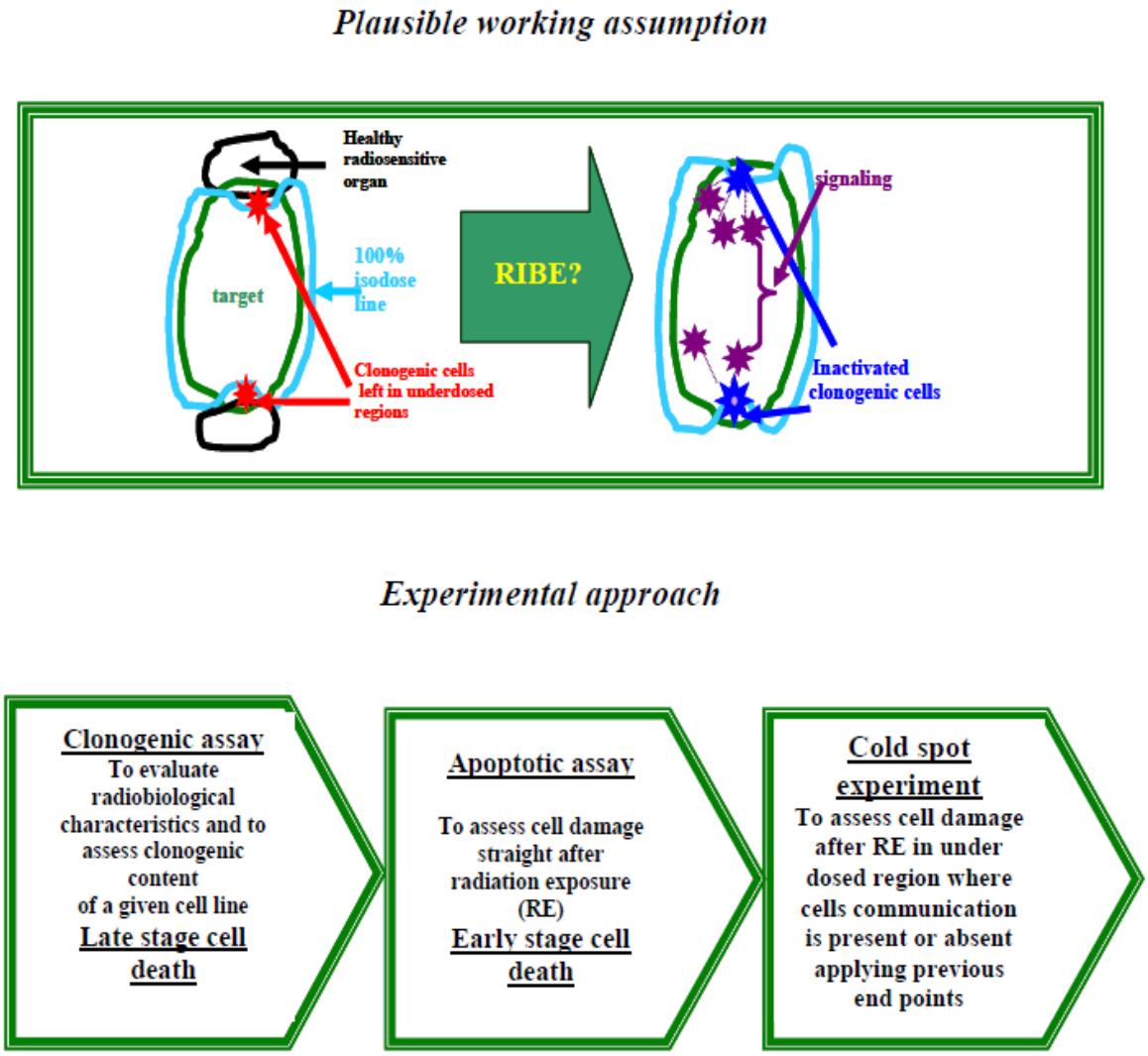

Figure 3.1. Schematic diagram representing experimental methodology used to investigate cellular survival in dose cold spot region.

It was assumed that dose cold spots in treatment plans do not lead to effects as significant as predicted by Tome [106], especially for very small volumes. This is because a small amount of clonogenic cells, left in underdosed region, could be inactivated as a result of inter and intra cellular signalling. To test this assumption the cellular response of both - freely communicating and non -

communicating cells after the delivery of a partly shielded radiation field were investigated. Before assessing cellular damage in an under-dosed region it was necessary to identify the type of cellular response to radiation treatment that can be observed in a given cell line. Published literature reveals many radiobiological approaches that are used to investigate RIBE, which involve different types of radiation treatment and by applying various biological assays [110]. In this work we analysed the early and late stages of the cellular damage by assessing apoptotic and clonogenic cell deaths, with the intention to apply these end points to investigate cell survival in a dose cold spot.

3.4. Materials and methods.

3.4.1. Cell culture.

The PC3 human prostate epithelial adenocarcinoma cell line, which was initially established from a patients' bone marrow metastasis [114], was kindly donated by Prof Wayne Tilley (the Dame Roma Mitchell Cancer Research Laboratories, University of Adelaide and the Hanson Institute). Cells were cultured as a monolayer in RPMI 1640 (Sigma) cell growth medium, supplemented with 10% foetal calf serum (FCS) (Gibco), 1% L-Glutamine, 1% sodium pyruvate, 1% ascorbate, 1% penicillin/streptomycin at 37^{o}C in a humidified atmosphere containing 5% /CO_2. From preliminary experiments, the colony forming efficiency and doubling time of the PC3 cell line were established and applied to all experiments discussed. The numbers of mock irradiated cells were plated to yield the survival rate established in the preliminary experiments.

3.4.2. Clonogenic assay.

This assay analysed the lethal effect of cytotoxic agents, such as ionising radiation, and investigated the cell growth potential for the given PC3 cell line. The main goal of clonogenic assays is to test a cell's capacity to produce sizeable colonies of descendants, which directly depends on the cellular functionality and reproductive integrity.

Published literature indicates a number of different methodologies to perform a clonogenic assay *in vitro* [115] [116] in terms of whether cells become detached or not after radiation treatment. In this study the former method was used as initially cells were seeded at high densities to achieve relatively high cellular confluence during radiation treatment, and later cells were replated at low densities for colonies to grow. The advantage of treating densely populated cells is in close match to physiological *in vivo* conditions where cells are tightly packed. Additionally, this approach allows investigation of all types of radiation induced cellular damages including direct hit and inactivation induced by intercellular signalling according to the new paradigm on cell killing mechanism [117]. This last type of radiation induced cellular response has two main mechanisms of action [118]: either long range Distant Cell Signalling Intercellular Communication (DSIC), involving free radicals, or short range Gap Junctional Intercellular Communication (GJIC), occurring through gap junction intercellular channels for cells which are in direct contact with each other [119]. Consequently for each type of radiobiological assay performed in this work the aim was to maintain relatively high cellular densities during radiation treatment.

Approximately $1.3x10^6$ cells per flask were plated in T75 (Corning, USA) flasks 24 hours prior irradiation at 37^oC /5% CO_2. Prior to irradiation, growth media was replaced with phosphate buffered saline (PBS). Following irradiation, the PBS was removed by aspiration and cells were washed with Hanks balanced salt solution (HBSS) and harvested from flasks - released by incubation with trypsin for 5 min in $37C^o$/5% CO_2. Cells were resuspended in HBSS, containing 5% FCS and centrifuged

(4000 rpm, 4 C^o). Cell pellets were then resuspended in RPMI 1640 growth media. Cell number was determined using trypan blue exclusion. Cells were plated into 6 well dishes (Corning, USA), depending on the radiation doses received, ranged from 200 cells/well for control flasks and no more than 3300 cells/well for flasks receiving 8 Gy. Cells were cultured for two weeks according to the estimated doubling time in order to mature into colonies containing more than 50 cells per colony to exclude cells which have a limited growth potential. Growth media was replaced every 4-5 days. Colonies were then fixed with methanol, stained with crystal violet and clonogenicity was counted using a stereomicroscope.

The colony forming ability after radiation induced cellular damage was based on the calculated cell survival data. Cell survival fractions (SF) for each radiation treatment were estimated taking into account colony forming efficiency (CFE) and by applying the following ratio [120]:

$$SF = \frac{colonies\ counted}{cells\ plated} \times CFE \quad (3.1)$$

Cell survival for the PC3 cell line was estimated from two independent experiments using triplicate fold samplings and plotted on the logarithmic scale as a function of dose received plotted on a linear scale. 'The dose which kills half of the plated clonogene population, D50, was estimated from this graph (see Figure 4).

3.4.3. Apoptotic cell death assay.

The second aim of this project was to determine the level of radiation-induced apoptosis in the PC3 cell line, to assess early responses to cellular damage following radiation exposure, an important

factor that contributes to rapid cell death in androgen-dependant malignant prostate tissues This would enable the analysis of the correlation between early and late responses following radiation exposure. Comparability of clonogenic and apoptotis rates can contribute substantially to the assessment of the validity of the dose cold experiment. For that reason the short term assay was tested against the results of a more laborious but reliable clonogenic assay[121]. Following estimations of the late cell survival data for several doses (0-8Gy), the rate of early apoptotic cell death was quantified to determine correlation with the short term assay. This was estimated based on the distinct nuclear morphology assessed by the cells' internal structures and surface characteristics analysis, which was measured by the side scatter (SS). Increased SS indicates greater structures and increased numbers of granules, which are inherent to non-viable cells. Dead cells exhibit a rougher cell surface and which produces a higher SS.

Analysis of cellular morphology and associated apoptotic bodies was performed by using 7-AAD (7-Amino-Actinomycin D) staining. This dye can readily move across the cell membrane and bind to cellular DNA, providing a means for identifying those cells that became damaged and hence lost membrane integrity.

Logarithmically growing PC3 cells were plated at the appropriate cell densities (approximately $0.5x10^6$ cells per well) into 6-well flasks and irradiated 24 hours later with 0, 2, 4 and 8 Gy. Following radiation exposure the flasks were placed into the incubator at $37^oC/5\%$ CO_2 and cultured for 24, 48 and 96 h. Cellular viability was assessed based on apoptotic cell morphology, with cells collected at each time point. Cells were washed twice with EDTA+PBS solution (1:50), harvested from flasks, centrifuged and resuspended in PBS. Following this, cells were stained with 1 mg/ml 7-AAD dye per 1ml of cell solution (1:100 dilution), removed from light, and incubated on ice for a minimum of 15 minutes to let dye to penetrate. Data for 10^6 cells per sample was acquired a with

FACScalibur™ flow cytometer (Becton Dickinson, USA) and analysed using CellQuest software (Becton Dickinson, USA), with two replicates per sample.

3.4.4. Dose cold spot experiment.

The aim of this experiment was to observe and analyse the previously investigated cellular responses after radiation induced damage in the underdosed region with and without intercellular cellular communication. To achieve this, five different types of irradiation treatments were performed by applying 2 Gy as the prescribed dose:

- sham irradiation (called "control")
- 100% of 2 Gy, delivered to non communicating cells - called "open field"
- 80% of 2 Gy delivered to non communicating cells -called "shielded"
- 100% of 2 Gy delivered to communicating cells - called "flask"
- 80% of 2 Gy delivered to communicating cells - called "slide".

For "control ", "open" and "shielded" radiation treatment a large amount of cells ($7x10^4$ cell/cm^2) was plated into each Peel-Off Tissue Culture Flasks (TPP, Techno Plastic Products AG, Switzerland) and incubated for 48 hours. Cell ability to grow on different plastic wares was tested and did not indicate any differences. For "flask" and "slide" radiation treatments one peel-off flask was used, which had required additional preparation before the same amount of cells ($7x10^4$ cell/cm^2) was plated into this flask. First, a very thin glass slide (5 cm x 2.1 cm and thickness of less than 0.1 cm), sterilised in pure ethanol, was inserted into peel-off flask using long tweezers and positioned in the middle of the flask. After that, the complete RPMI-1640 cell growth media was carefully added into the peel off flask to avoid slide movements. This was followed by cell injection and very careful flask transportation into an incubator.

Flasks were incubated for 48 hours in order to allow cells adhesion and to let the slide become attached to the flasks' surface due to the tension forces and uniform cell growth on the top of the slide.

Prior to irradiation the media in each flask was replaced with PBS. Following this, the cells were treated with 100% and 80% of the prescribed dose respectively by applying appropriate shielding where needed. The dose cold spot in freely communicating cells was created by placing 6 mm thick lead sheet to fully cover the cells growing on the slide. The dimensions of the applied shielding material (50 mm x 21 mm x 6 mm) reduced the prescribed dose by 20%, which was delivered to an average 9% of the total cell population treated in the flask. Concurrently, the "open field" and "shielded" types of radiation treatment were performed in the two separate flasks. It was accomplished by delivering 2 Gy open field to one flask while the second flask was fully covered with 6mm thickness lead sheet to deliver 80% of the prescribed dose (see Figure 3.2). The radiation induced response in non-communicating cells treated with opened and shielded radiation fields was compared to cellular responses obtained from communicating cells treated with the same amounts of radiation by applying spatially non-uniform radiation beam.

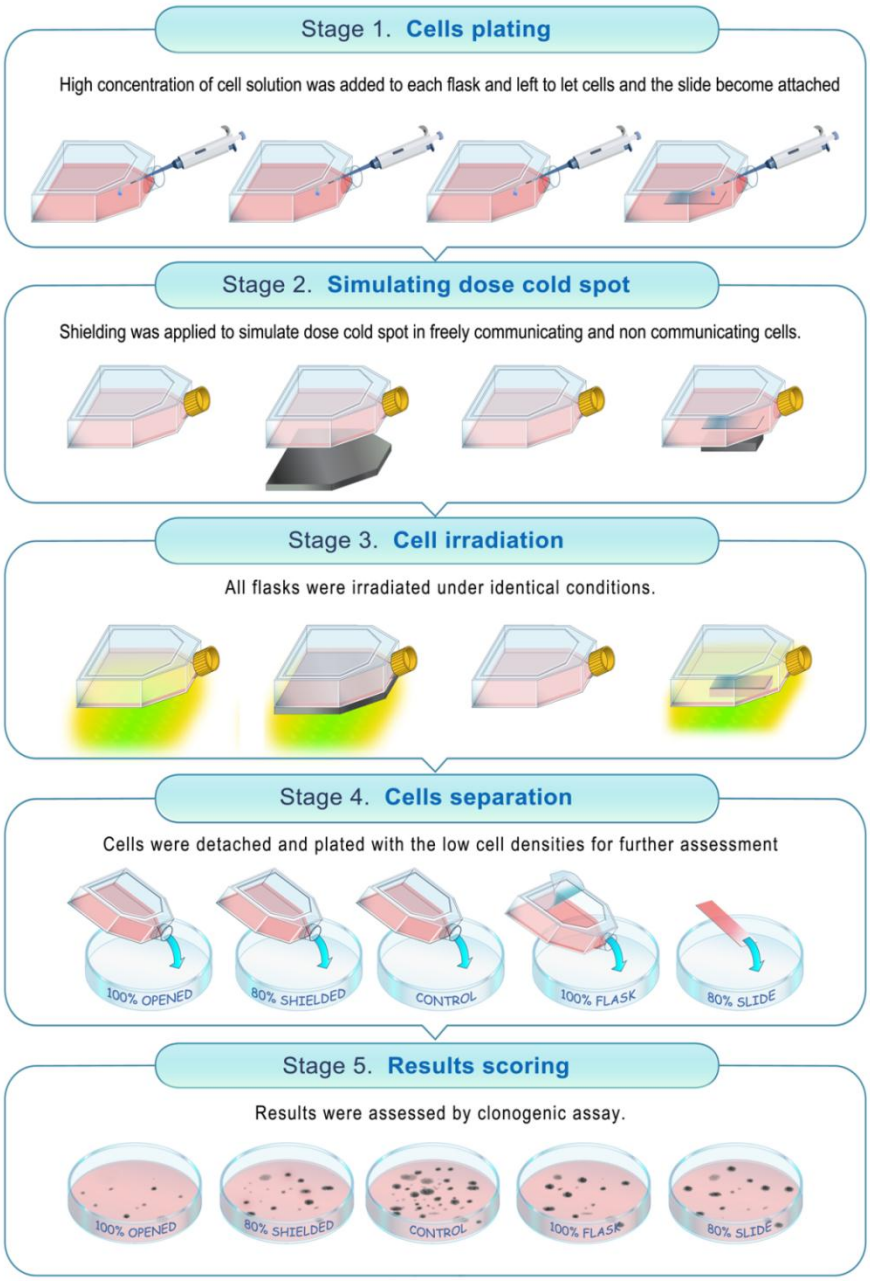

Figure 3.2. Stages of dose cold spot experiment performed applying five different types of radiation treatments: non-irradiated control, 'shielded' cells received 80% of the prescribed dose, 'open' cells received 100% of the prescribed dose separately from shielded cells, 'slide' and 'flask' cells received 80% and 100% of the prescribed dose in the same flask.

After treatment, cells were incubated for two hours to allow intercellular communication and were then harvested as described for the clonogenic assay. Cells growing on the slide were similarly harvested after the slide was carefully removed from the flask. Cells were washed, resuspended, replated for the clonogenic assay and incubated for two weeks as described previously.
This experiment was repeated twice with triple samplings.

Results were scored based on the doubling time, 14 days after cells irradiation, and were analysed in Graph Pad Prism 5 software **(**GraphPad Software, Inc., CA, USA) with one way Anova analysis of variances followed by Tukey's Multiple Comparison Test. This statistical approach is different from a regular t test as it considers the scatter of all groups, giving the test more power to detect differences. Furthermore multiple comparisons allow evaluation of the significance level considering the entire family of comparisons (10 in this experiment) instead of each comparison individually. This means that if all the groups have the same mean, there is only 5% probability that any one of the comparisons would reach a statistically significant conclusion randomly. This is usually achieved at the cost of applying a stricter significance threshold [122].

3.4.5. Cell irradiation set-up and validation of irradiation procedure.

Cell irradiations for clonogenic assay, apoptotic assay and dose cold spot experiments were performed at the Radiation Oncology Department, Royal Adelaide Hospital, using a 6 MV x-ray beam produced by a Varian iX linear accelerator (Varian, Palo Alto, CA, USA). Clinically applied nominal dose rate of 3 Gy/min was used. The flasks were placed on top of 1.5 cm thick solid water build up sheets (RW3, ρ=1.0459g/cm^3, PTW, Germany) to ensure that the cell line was positioned at the depth of maximum dose. Cells were treated posteriorly with gantry positioned at 180^o (as cells were adhered to the bottom flask surface) (see Figure 3.3).

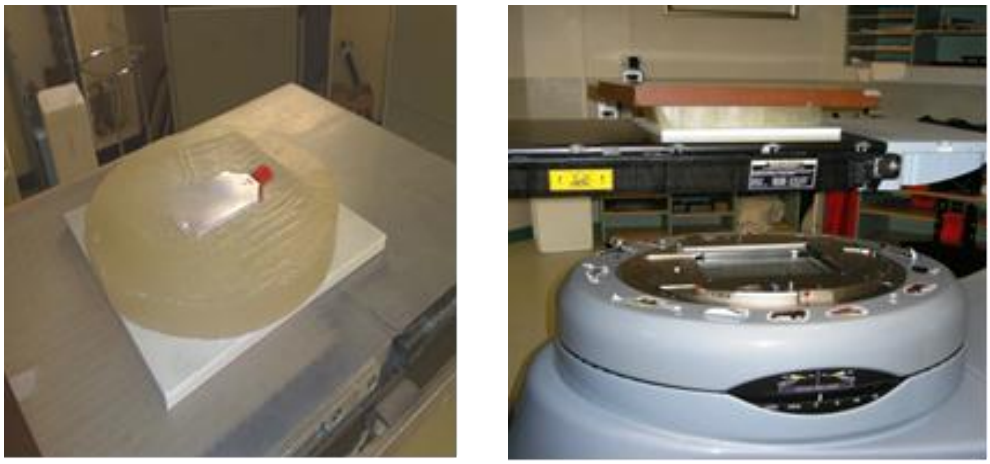

Figure 3.3. Cell irradiation experimental set up which demonstrates flasks being irradiated posteriorly with 1.5 cm solid water build-up sheet, custom made wax phantom and thick solid water slab was put on top of the flasks ensure full scatter conditions.

In each case, radiation treatment was performed with the build-up surface at 100 cm from the beam focal spot and with 20 x 20 cm^2 radiation field size. To minimise the effect of an air inhomogeneity on dose distribution and possible changes in scatter radiation due to an air gap above the cell monolayer surface, flasks were fully filled with PBS. Flasks were inserted in custom made wax phantoms (for different flask sizes) and 5 cm thick solid water slab was put on top of the flasks to ensure full scatter conditions. Accelerator radiation output, calibrated using IAEA TRS 398 protocol [123] was routinely checked with Daily QA 3™ device (Sun Nuclear, USA) prior to all radiation treatments. The beam-on-time parameters, so called monitor units (MU), were determined using chip LiF100 thermoluminecent dosimeters (TLDs). TLDs were irradiated using the same experimental setup, (i.e. they were positioned inside flasks at the location of the cell monolayer) to determine the MUs required to deliver 2 Gy absorbed dose to the cells. In the case of dose cold spot experiments, the thickness of lead shielding, which results in 20% dose reduction, was determined with farmer type NE 2577 0.2 cm^3 ionisation chamber (Nuclear Enterprises, England). Once the

required lead thickness was found, it was again confirmed using LiF100 thermoluminescent chips; i.e. water-proofed TLD chips were placed on the beam central axis between flask's bottom surface and lead shielding. Flask was filled with water and irradiation was performed posteriorly using same 1.5 cm thick solid water build up sheets. The results confirmed dose reduction by 20%. In addition, gafchromic RTQA2 film (International Specialty Products, Wayne, New Jersey, USA) was positioned underneath the flask. The dose map obtained confirmed the position and dimensions of the cold spot as well as the sharpness of the penumbra between 100 and 80% dose regions.

3.5. Results.

In this study we investigated cell survival by analysing the early and late responses of cellular damage induced by ionising radiation with or without intercellular communication. These results and discussion are presented in the following sections.

3.5.1. Clonogenic survival and radiobiological characteristics of PC3 cell line.

It was found that for the given cell line under existing cell growing conditions the population doubling time was 48 hours and the average CFE was 0.60 (±0.027).

In the current work the mean survival fractions (Table 3.1) from two independent sets of experiments with three and six fold samplings were calculated by using equation (1) and fitted by a curve (Figure 3.4) according to the linear-quadratic relationship [124].

Dose (Gy)	SF	SE
1	0.800	(± 0.0212)
2	0.586	(± 0.0279)
3	0.304	(± 0.0343)
4	0.137	(± 0.0116)
5	0.050	(± 0.0069)
6	0.022	(± 0.0035)
8	0.004	(± 0.0006)

Table 3.1. Mean cell survival fractions and associated standard error values from two independent experiments using triplicate and six fold samplings.

From the cell survival curve, which generally represents the sensitivity of certain cells to certain radiation treatments, it was possible to estimate the dose that killed half of the clonogen population (D50) as being 2.037 Gy. The fraction of cells which survived the standard clinical daily dose of 2 Gy (SF2) within curative radiation protocols was found to be 0.586 (± 0.0279).

Figure 3.4 comparatively illustrates PC3 cell survival data from this experiment comparing with literature extracted* data [115, 116, 125, 126] for the same cell line.

*Cell survival data from Deweese', Algan' and Lieth' experiments was extracted by Carlson *et al* [131] [132].

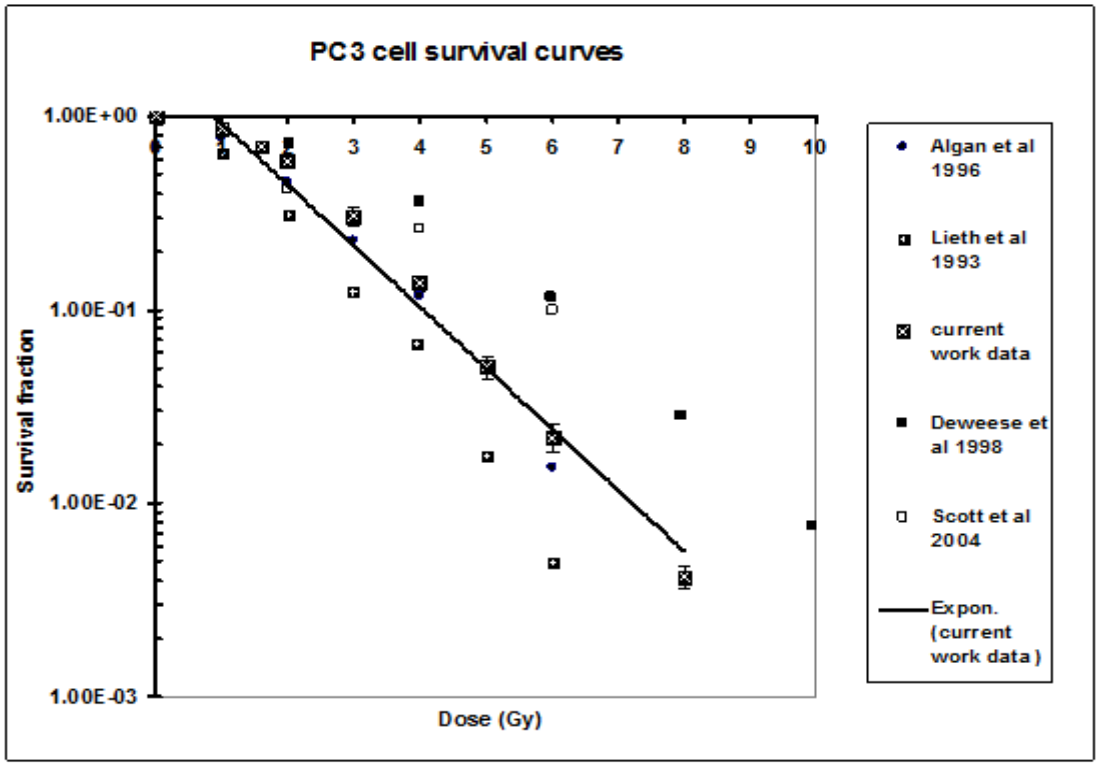

Figure 3.4. Cell survival curves reported in the literature as compared to current work.

A brief summary of some of these experimental settings is provided in Table 3.2. As expected, cell growing conditions and radiation treatment conditions play an important role in cellular radio-responsiveness, which varied significantly in *vitro*. This discrepancy is even more prominent among in *vivo* [127-130].

Reference	Type of Experiment	Proliferation Status	Radiation Source	Radiation Type	Dose Rate
Algan et al	colony survival assay	60-80% confluence	Cs-137 irradiator	0.661 MeV gamma	84 Gy/h
Deweese et al	colony survival assay	Sub confluent cultures	Cs-137	0.667 MeV gamma	60 Gy/h
Lieth et al	colony survival assay	exponentially growing	250 kVp x-ray machine at 250 kV and 15 mA		60 Gy/h
Current work	colony survival assay	high confluence	linear accelerator	6 MeV x rays	180 Gy/h
Joiner et al	colony survival assay	>70% confluence	linear accelerator	15 MeV x rays	360 Gy/h
Scott et al	colony survival assay	n/a	linear accelerator	6 MeV x rays	138 Gy/h

Table 3.2. Summary of some experimental set ups used to perform clonogenic assay using PC3 cell line.

The SF2 values reported in the literature for the same PC3 cell line are represented together with our data in Figure 3.5. These differences between published and observed values can be attributed to many factors such as: type of radiation, dose rate and dose protraction, beam quality, irradiation set ups including applied radiation field sizes and source to surface distance (SSDs), presence or absence of build-up material and presence or absence of air gaps etc.

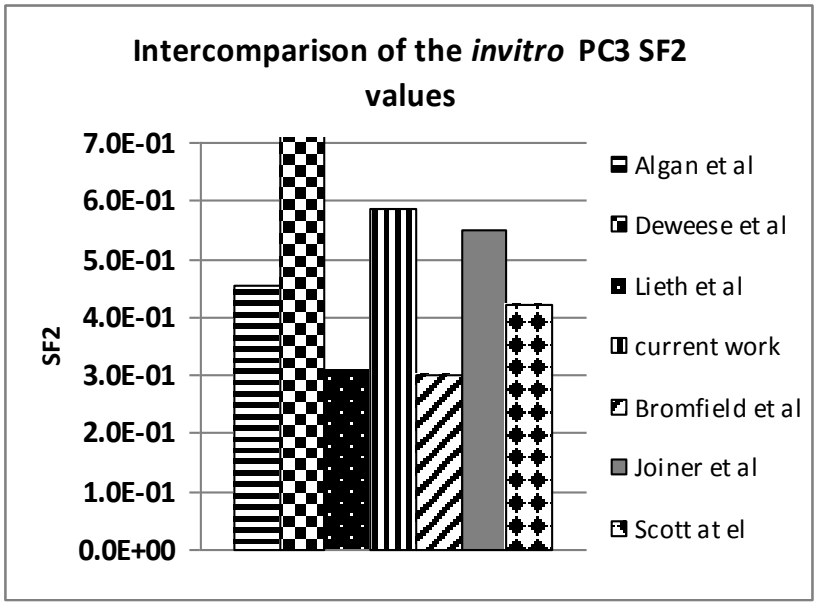

Figure 3.5. Variations in SF2 values reported in the literature for PC3 cell line compared to current work.

Other major factors, which has an impact on the way these cells respond to radiation treatment, is the intra-laboratory variations in cells growing conditions, origin of the cell stock, differences in the experimental protocols used to perform clonogenic assay and data analysis. Cellular distribution among the cell cycle and consequently cell radio-resistance is another acknowledged parameter which influences cellular radio-responsiveness [126, 133, 134]. Thus, SF2 value reported by Deweese *et al* was more than twice higher (131.58%) compared to Lieth *et al* data, despite the same

dose rate used. This significant increase in cell survival cannot be explained solely by the slight (-0.54%) variation in the treatments delivery time [132], or by differences in the type radiation applied (250kV x-rays versus 661 keV gamma rays), or even by the variation in the experimental protocols used to perform clonogenic assay (in terms of detaching cells after irradiation). Most possibly this difference arises from the multifactorial dependence of all experimental conditions.

The clonogenic analysis of the PC3 cell line allowed observation of late responses of radiation induced cellular damage and to identify SF2 and D50.

It was found that PC3 cells differ widely among laboratories and are very sensitive to the methods used to evaluate radiosensitivity parameters [131].

Thefore the variation in the radiobiological characteristics of the same PC3 cell line, cultured and treated under different conditions, clearly indicated the importance to evaluate D50 independantly rather than relying on the published data.

3.5.2. Negligible evidences of the apoptotic cell death in PC3 cell line.

Figure 3.6 shows an inter-comparison between the lowest and the highest SS observed at the 48^{th} and 96^{th} time collection points since there was not any measurable (> 1%) cells death detected 24 hours after radiation treatment. In fact, there was not any significant dose responsive (see Figure 3.7) cells death observed until 96 hour radiation exposure. Following this time surviving cells start to demonstrate their colony forming abilities [135] and first observable survival colonies can be detected.

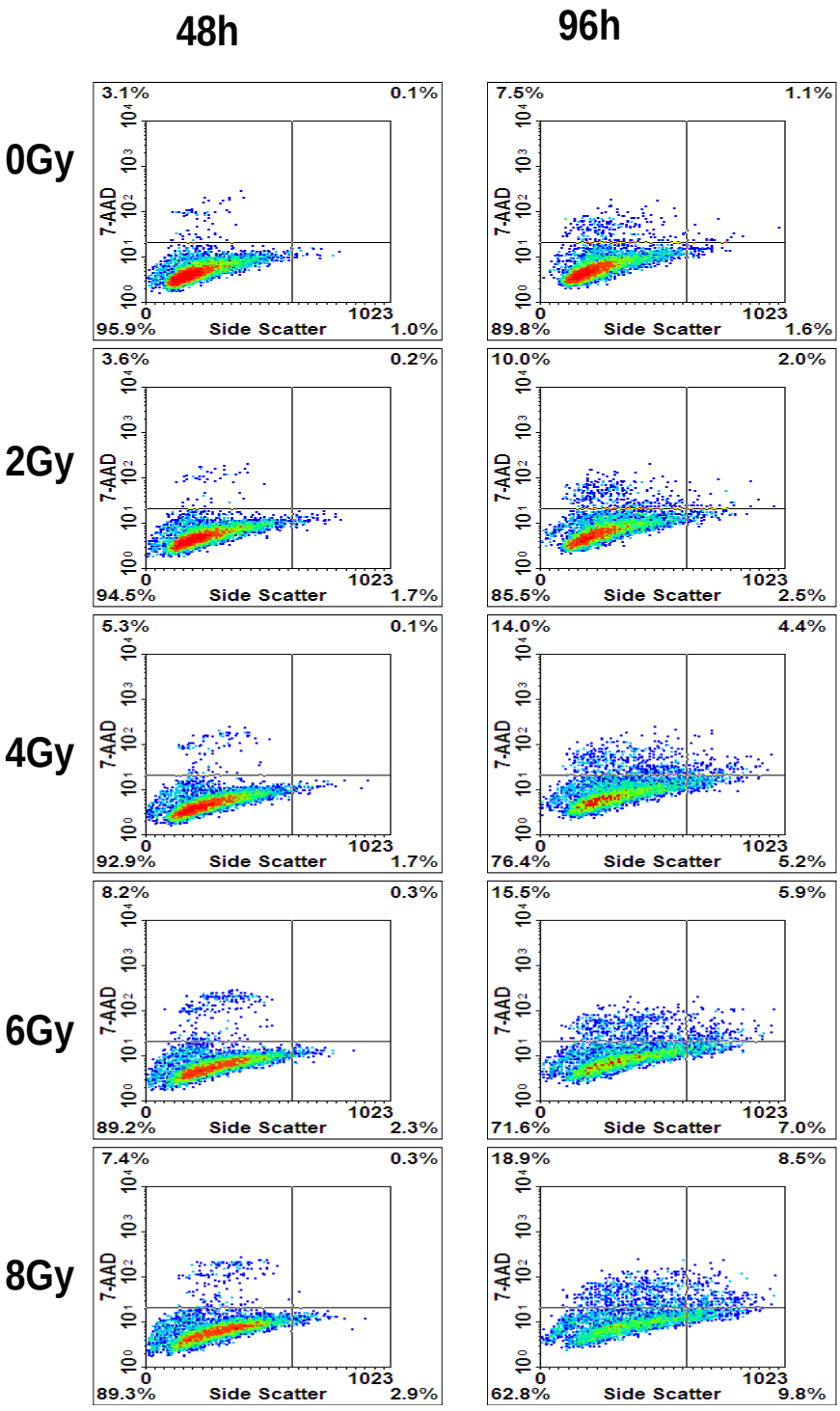

Figure 3.6. Analysis of cellular morphology and associated apoptotic bodies. Results are plotted as density plots representing PC3 cells labelled with 7AAD against side scatter 48 and 96 hours after radiation exposure. Top two quadrants represent apoptotic bodies (in blue). Morphologically normal cells are seen in the lower right quadrant (all other colours).

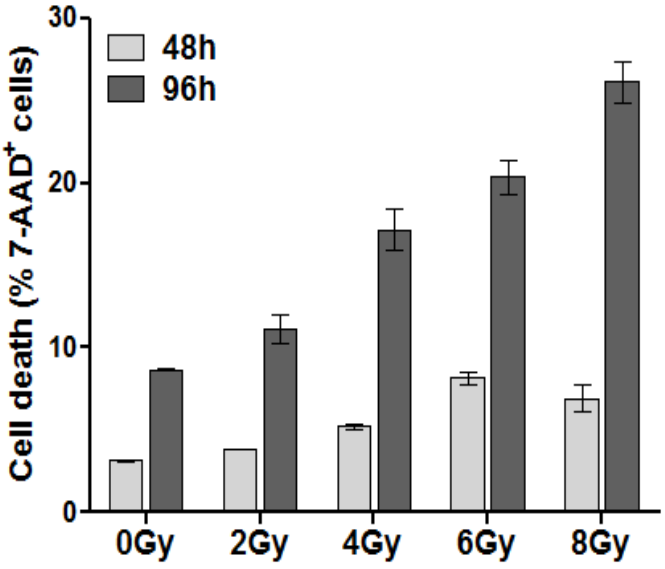

Figure 3.7. Percentage of apoptotic cell bodies labelled with 7-AAD$^+$ detected with FACScalibur™ flow cytometer in PC3 cell line at 48 and 96 hours after radiation exposure.

Nevertheless, at the 96th time collection point there was an increase in cell death up to 30%. This result cannot be explained as an apoptosis via typical apoptotic pathway, as this occurs relatively quickly just prior to the first post-irradiation mitosis, in cell lines where apoptosis is the primary mode of cell death [136] .

The observed increase in SS was more likely triggered by the radiation-induced premature replicative cellular senescence, which is also morphologically characterised by cytoplasm flattening and increased cells granularity[137] [138]. An increased number of the senescence like morphological bodies was observed in the current experiment during routine microscopic examination at the late stages after radiation exposure.

This result is in agreement with data reported by Bromfield *et al* [105] who also observed the minimal amount of apoptotic deaths in PC3 cell line and concluded that an apoptotic cell death as a

rapid reaction to ionising radiation is not the way PC3 cells respond. This is a response one would expect in the cell line with mutated p53 protein, which controls cell cycle arrest and apoptosis by halting the cell cycle in G1, while DNA damage is present.

Alternatively, another possible explanation for the minimal apoptotic cell death observed, according to Kyprianou, N. *et al*, Sheridan, M.T. *et al* , Lock et al and Tannock I. *et al* [139-142], is that the apoptosis pathway may be the primary death mode after low LET radiation exposure only in particular cell types, such as hematopoietic and lymphatic, but not in epithelial and stromal cell lines. It appears that these cells primarily die due to reproductive cell death by inducing permanent growth arrest and replicative senescence, which is efficient enough in removing cells from clonogenic pool without activating apoptosis. Our results are in an agreement with previous experimental work [139-141] showing that radiation induced apoptosis does not have an impact on the clonogenic survival, which suggests that there is no correlation between apoptotic and clonogenic cell death pathways in certain epithelial cells lines after radiation exposure.

In summary, there was no significant dose dependent early cell death observed in the current work until up to 4 days after radiation exposure – a time when first sizable colonies can be detected by the clonogenic survival assessment. The minimal observed level of the radiation induced apoptosis indicates that for the given PC3 cell line in existent growing and treatment conditions, the apoptotic cells death is not the main response pathway to radiation exposure and these cells primarily die by reproductive cell death.

3.5.3. Experimental evidence of the decreased cell survival in freely communicating cells in dose cold spot region.

The ability of cells to form colonies after cellular DNA damage, which was assessed previously with the clonogenic assay, measures the long-term survival data. This summarises all radiation

induced cellular lethal damages including early apoptosis; mitotic catastrophe, leading to late apoptosis or necrosis; permanent growth arrest, followed by necrosis; and cells' senescence [121, 143]. According to this and taking into account the minimal level of apoptosis in PC3 cell line that did not correlate with the clonogenic assay results, the latter was used to assess cellular damage after radiation exposure to 80% of 2 Gy radiation dose in the regions where cell communication was either present or absent.

Statistically significant (P<0.001) [144] decrease of up to 42.2% in cell survival was found between non-irradiated "control" group and cells from the "open" flask receiving 100% of the prescribed 2 Gy dose (see Table 3.3).

Test No.	**Comparison Test**	**Mean Diff. Survival**	**Significant? P < 0.05?**	**Summary**	**95% CI of Diff**
1	Control vs Open	42.2	P<0.001	***	27.2 to 57.2
2	Control vs Shielded	20.2	P<0.01	**	4.5 to 35.8
3	Control vs Flask	41.6	P<0.001	***	26.7 to 56.6
4	Control vs Slide	36.4	P<0.001	***	21.4 to 51.4
5	Open vs Shielded	-22.0	P<0.01	**	-37.0 to -7.0
6	Open vs Flask	-0.5	P>0.05	ns	-14.8 to 13.8
7	Open vs Slide	-5.8	P>0.05	ns	-20.1 to 8.5
8	Shielded vs Flask	21.5	P<0.01	**	6.5 to 36.4
9	Shielded vs Slide	16.2	P<0.05	*	1.2 to 31.2
10	Flask vs Slide	-5.3	P>0.05	ns	-19.5 to 9.0

Table 3.3. One-way ANOVA analysis of variances with Post Tukey's Multiple Comparison Test of the PC3 cell survival in dose cold spot experiment. In Graph Pad Prism 5 software the level of statistical significance can be chosen between 0.05, 0.01, or 0.001, which graphically presented as *, ** and *** symbols respectively.

This was in agreement with the above mentioned SF2 value of 0.586 (± 0.0279) determined by clonogenic assay. Furthermore, cell survival of cells from "open" and "shielded" flasks that received doses of 2 Gy and 80% of 2 Gy, respectively, was also found to be significantly different ($P<0.001$). This indicates that cells which received 100 % of the prescribed dose and cells under-dosed by 20% responded differently to doses delivered, when irradiated separately (see Figure 3.8).

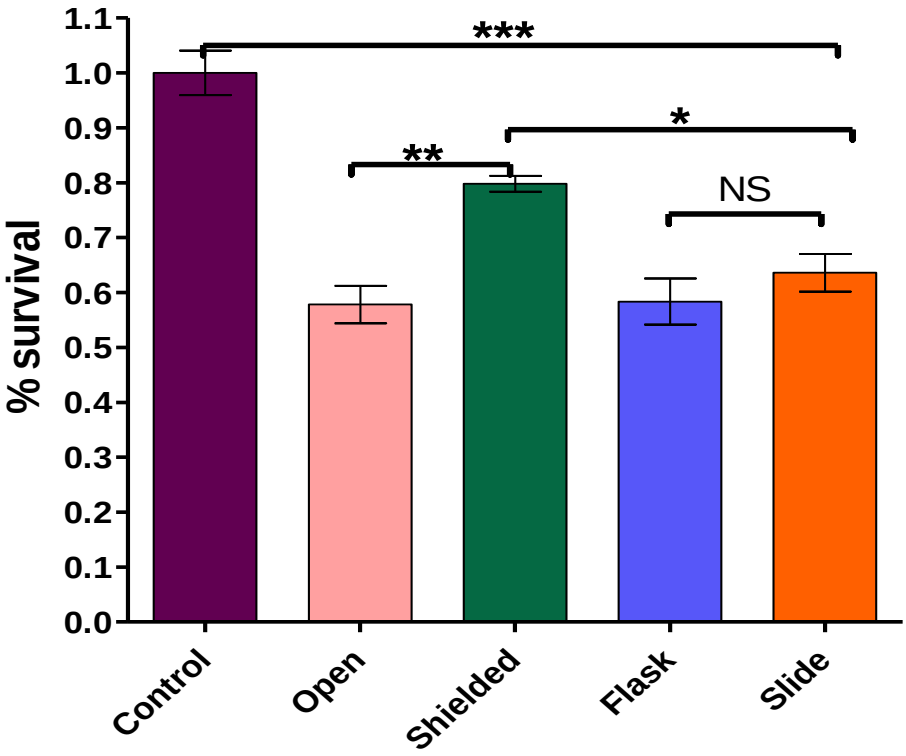

Figure 3.8. PC3 cell survival analysis after radiation induced damage in the underdosed region with and without intercellular cellular communication. 'Shielded' and 'open' columns represent cell survival after receiving 80% and 100%of the prescribed dose separately. 'Slide' and 'flask' columns demonstrate survival data in cells which were in direct contact with each other and treated with 80% and 100% of the prescribed dose. In Graph Pad Prism 5 software the level of statistical significance can be chosen between 0.05, 0.01, or 0.001, which graphically presented as *, ** and *** symbols respectively.

However, survival comparison between cells from "flask" and "slide" positions within the flask exposed to the modulated radiation field (with both groups of cells being in direct contact during and shortly after irradiation) did not indicate any statistically significant ($P>0.05$) difference even though the delivered radiation dose also differed by 20%.

On the other hand, there was a significant ($P<0.001$) decrease in cell survival observed for "control" versus "flask" cells (41.6%) and "control" versus "slide" cells (36.4%). This indicated that communicating cells treated with 100% and 80% of the prescribed 2 Gy dose responded similarly (only 5.2% mean difference). These results are in contradiction with cell survival data from “open” and “shielded” flask irradiation groups (i.e. the same radiation doses applied to non-communicating cells) where the mean survival difference was 22.0%.

As mentioned above, survival decrease between cells from non-irradiated "control" and "flask" (100% of the prescribed 2 Gy dose) groups was 41.6%. This correlated well with the mean survival difference of 42.2% between "control" and "open" cell groups, treated with the same doses.

The mean difference of 16.2%, on the other hand, in survival between cells from “shielded” and “slide” groups (both exposed to 80% of the prescribed dose) was found to be statistically significant ($P<0.05$); i.e. higher cell kill was observed in under-dosed cells when they were directly communicating with cells exposed to 100% of the prescribed dose.

In addition, using the measured cell survival curve (see Figure 2), the survival fraction corresponding to 1.6 Gy (i.e. 80% of 2 Gy) radiation dose was estimated to be 0.70. The survival fraction of cells from the “shielded” flask irradiated with 1.6 Gy was found to be 0.79. These values agree within ± three standard deviations and therefore are not considered to be significantly different.

3.6. Discussion.

It was hypothesized in the current work that radiation induced bystander killing caused by intercellular signalling may have an impact on cell survival of the PC3 prostate cancer cells in dose cold spots. To test this assumption, cellular capacity to produce sizeable colonies of descendants after radiation exposure was assessed by clonogenic assays. Clonogenic assay method was also used as the measured endpoint to quantify the extent of radiation damage as it includes all radiation induced cell death modes. However, initially the early responses of cellular damage were also investigated in the PC3 cell line. This would enable the analysis of the correlation between early and late responses following radiation exposure. Evaluation of clonogenic versus apoptotic rates could contribute substantially to the assessment of the validity of the dose cold experiments. For that reason the short term assay was tested against the results of a more laborious but reliable clonogenic assay. However, this investigation did not reveal any significant cell death through radiation-induced apoptosis. The obtained result is in agreement with the data reported by Bromfield *et al* [105] who also observed minimal amount of apoptotic deaths in PC3 cell line and concluded that an apoptotic cell death as a rapid reaction to ionising radiation is not the way PC3 cells respond. Additionally our results are in agreement with previous experimental work [139-141] showing that radiation induced apoptosis does not have an impact on the clonogenic survival, which suggests that there is no correlation between apoptotic and clonogenic cell death pathways in certain epithelial cell lines after radiation exposure. Consequently cell survival determined from the clonogenic assay was used as measurable endpoint to quantify an extent of the damage as it summarises all radiation induced cell death modes.

The literature revealed the controversies in terms of prostate radio-responsiveness, which can widely differ even within the same cell line. It was found that PC3 cells differ widely among

laboratories and are very sensitive to the methods used to evaluate radiosensitivity parameters [131].These detected variations in prostate radio-sensitivity *in vitro,* reported for the same cell line from the different laboratories, acknowledged the necessity to investigate these parameters independently instead of relying on the published data

In vivo prostate radio-sensitivity derived from the clinical data still remains to be controversial in terms of reported values which vary up to a factor of two. This indicates the differences in prostate treatment radio-responsiveness within the patient pool. Moreover, radio-sensitivity can be altered within the same patient throughout radiation treatment as a result of redistribution and reoxygenation processes [145].

Inter-patient and inter-laboratory variations in prostate radio-responsiveness emphasize the necessity of individualised treatment planning and highlight the limitation of directly using *in vitro* evaluated parameters to predict clinical outcome. This also indicates the compulsion of not being conclusive towards clinically applied procedures based solely on the *in vitro* data.

It has been demonstrated in the current work that survival of PC3 prostate cancer cells (for given growing and treatment conditions) was significantly different for under-dosed cells that were either in physical contact with cells receiving 100% of the prescribed 2 Gy dose or irradiated separately.
It has been concluded that reduced survival in under-dosed cells, when cells were in contact with cells irradiated with full 2 Gy dose, was most likely a result of intercellular communication and the ability to share the same stress chemical messengers (that mediate this communication, such as hormones, cytokines, growth regulators, etc.) secreted by irradiated cells. This conclusion has been based on the fact that cells growing on the “slide” were treated with the same amount of radiation dose (80% of 2 Gy radiation dose), plated at the same densities and maintained in the exact same conditions as cells also irradiated with 80% dose but irradiated separately from the cells that received

100% dose. It could therefore be reasoned that intercellular communication might have contributed to the decrease in cell survival of cells collected from the "slide".

Communication between cells is important for maintaining homeostasis, i.e. the physiological regulatory processes that keep the internal environment of a system in a constant state. It allows cells to sense changes in their microenvironment, to integrate internal or external signals, to respond to them by changes in transcriptional activity, metabolism, or other regulatory measures. Signalling pathways generally consist of widespread building blocks, such as receptors, extracellular signal-regulated kinase (ERK) or mitogen-activated protein kinase (MAPK) cascades, G proteins and small G proteins such as Ras, Rho, Rab, Ran, or Arf, etc [146]. Signalling can be seen as a linear connection between input elements (the receptors) and output elements (such as regulators of gene expression). However, there is evidence that signalling pathways interact with each other, forming a network. In spite of their variety in function and design, many signalling pathways usually consist of the same essential components, which are often highly conserved through evolution and between species.

Investigations of specific signalling pathways and possible underlying mechanisms responsible for the observed variation in radio-responsiveness in dose cold spot regions were out of the scope of this study. However, it is likely that the variation was caused by increased amount of cytotoxins secreted into the culture medium by cells receiving higher radiation dose or by substances passing through gap junctions from the directly affected cells to neighbouring cells causing additional damage in under-dosed cells [147]. There is experimental evidence that cytokines, i.e. cell-signalling protein molecules, play a major role in the cellular response to radiation and can greatly affect intrinsic cellular radiosensitivity as well as the incidence and type of radiation tissue complications [148, 149] [150].

Extensive work investigating non-targeted effects of ionising radiation implied that there were several signalling mechanisms responsible for this phenomenon. Burr *at el* suggested a few signalling pathways involving FasL, TNF-alpha, nitric oxide (NO), reactive oxygen species (such as superoxide and hydrogen peroxide) and macrophages as a source of DNA damaging signals [151]. Hei *at el* investigated the inhibition of the ERK and confirmed the involvement of the MAPK signalling cascade in the bystander process [152, 153]. MAPK pathways were associated with growth factor-mediated regulation of cellular proliferation, senescence, differentiation and apoptosis. Furthermore, Hei *at el* provided evidence that NO and calcium signalling are part of the signalling cascade. Work of Zhou *at el* demonstrated that the cyclooxygenase-2 (COX-2)-related pathway (which mediates cellular inflammatory response) was the crucial signalling link in the bystander phenomenon [2].

It is plausible that some of the literature-reported signalling pathways might be involved in the decreased cell survival observed in this work.

The main emphasis of the current work was to investigate whether there is any impact of non-targeted additional cell killing induced by intercellular communication in dose cold spot. We were able to quantify, for the current experimental set-ups, the magnitude of survival reduction in under-dosed cells that were in direct contact and shared same environmental media with cells receiving higher radiation dose. Our results agreed with previously reported 10-30% survival reduction [154, 155] and indicated involvement of factors that were previously associated with the radiation-induced bystander effects after exposure to modulated radiation fields or to irradiated cell conditioned medium.

To date, there have been several studies reported in the literature investigating cell survival in modulated radiation fields as intensity modulated radiation therapy (IMRT) is at present routinely used in many health institutions. For example, Bromley et al [156] investigated spatial distribution of

cell growth after irradiation with modulated x-ray intensity pattern. The work aimed to develop a method which could be used for in vitro investigation of changes in cellular radiation response associated with IMRT techniques. Later, the same research group presented two methods of predicting the number of cells that would survive modulated x-ray irradiation [157]. They reported differences between the measured and predicted surviving fraction of A549 cells following exposure to modulated x-ray fields: when the x-ray fluence map produced a steep dose gradient across the sample, fewer cells survived in the non-irradiated region than expected; when the x-ray fluence map produced a less steep dose gradient across the sample, more cells survived in the non-irradiated region than expected.

More recent studies of Butterworth *et al* [158] and Trainor C *et al* [159] used modulated field irradiation and cell separation techniques similar to those of Suchowerska *et al* [62] and Bromley *et al* [157]. Both studies investigated responses to steep dose gradients across populations of cells and reported significant decreases in cell survival for cells outside the primary radiation fields. Butterworth concluded that out-of-field effects were important determinants of cellular response following exposure to modulated irradiation fields and that survival could be affected by cellular communication between differentially irradiated cells. These experiments were performed using sparsely seeded cell cultures. Therefore the RIBE could have only occurred through release of soluble factors from irradiated cells.

In the current work non-uniform irradiations were performed directly within the primary field of the 6 MV x-ray beam, quality of which (in terms of low energy component of the fluence spectra) is different from the penumbra regions [158] used in studies of Butterworth *et al*, Trainor C *et al*, and Bromley *et al*. In the present study we investigated the worst possible scenario (from the clinical point of view) by simulating a dose cold spot on the central axis of the primary beam. Additionally, in this work novel cell separation technique was used. Unlike other experimental works investigating

cell survival in modulated radiation fields [157, 159], in this study cells were initially seeded at high densities to achieve relatively high cellular confluence during radiation treatment, and later cells were replated at low densities for colonies to grow. The advantage of treating densely populated cells is the close match to physiological in vivo conditions where cells are tightly packed. Additionally, this approach allows investigation of all types of radiation induced cellular damages including direct hit and inactivation induced by intercellular signalling.

Our results, obtained from two independent sets of experiments with sixfold samplings for the first set of experiments and threefold samplings for the repeated set of experiments, clearly indicate that for the PC3 cell line used and for given growing and irradiation conditions, there was statistically significant decrease in cell survival in dose cold spots, presumably due to intercellular communication. However, our findings do not imply that one can clinically ignore a dose cold spot in treatment planning and rely on intercellular signalling, which may or may not inactivate an unknown amount of clonogens. The mechanisms behind radiation-induced additional cell killing are still not clear. Little is also known about the types of DNA damage affecting bystander cells and whether irradiated cells have persistent ability to generate bystander signals.

3.7. Conclusion.

From clinical point of view, the impact of non-targeted effects of ionizing radiation in novel radiotherapy treatment techniques, such as intensity modulated radiation therapy and tomotherapy, requires further investigation as these techniques deliver highly conformal but modulated doses to tumours and also cover bigger tissue volumes with lower integral doses where bystander responses are more pronounced [160]. It is also possible to contemplate that if RIBE *in vivo* can be controlled and modulated, it may contribute to development of novel therapeutic approaches to cancer treatment

and to improve the existing ones. However, its clinical relevance in radiotherapy remains yet to be elucidated.

Chapter 4.0

Experimental investigation of the cytotoxicity of medium-borne signals in human prostate cancer cell line.

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4.1. Abstract.

Evidence exists that exposure of non-irradiated cells to Irradiated Cell Conditioned Medium (ICCM) can cause effects similar to those resulting from direct radiation damage. This study attempts to validate the stochastic model, relating absorbed dose to the emission and processing of cell death signals by non-irradiated cells, *in vitro* in PC3 human prostate cancer cell line.

The recipient cell survival was measured after exposure of cells to ICMM derived from donor cells: a) exposed to radiation doses from 2 to 8Gy and b) of concentrations varying from $2x10^2$ to $6x10^6$ irradiated with 2Gy.

Exposure to ICCM, irradiated with doses between 2-8Gy, resulted in a significant ($P<0.001$) decrease in clonogenic survival of non-irradiated recipient cells compared to the control group. However, dose dependency above 2Gy was not observed, indicating that any dose threshold was below 2Gy. A significant ($P<0.001$) decrease in survival was found in recipient cells exposed to the ICCM, derived from different concentrations of donor cells exposed to 2Gy, compared to the control

group. The recipient cell survival following exposure to ICCM derived from $2x10^2$ cells was significantly higher ($p<0.5$) compared to the rest of donor cell concentrations, indicating that the toxicity of ICCM depends on the cellular concentration of donor cells.

Non-linear regression data fitting provided reasonable agreement with the microdosimetric model for the induction of cell killing through medium-borne signals.

For the given cell line and given experimental conditions, significant decreases in cell survival were observed in non-irradiated cells exposed to ICCM derived from donor cells of various concentrations and irradiated with different doses.

4.2. Introduction.

It has been established, mostly in low-dose [161], microbeam [162, 163], modulated radiation [154] and medium transfer experiments [164], that cells directly hit by radiation may affect cells not damaged by radiation through factors transmitted via extra-cellular matrix [111] or through gap junctions [41] between cells. Intercellular signals may reach these cells (also known as a bystander cells) and produce many of the same effects as direct radiation damage.

Experimental evidence [18, 165] indicates that cells directly exposed to low linear energy transfer (LET) radiation release a transmissible cytotoxic factor(s) the into cell growing medium that can be transferred to non-irradiated cells.

It has been demonstrated [59] that exposure to medium from irradiated donor cells, referred to in the literature as Irradiated Cell Conditioned Media (ICCM), may affect recipient cells that are not directly exposed to radiation. Mothersill and co-workers [164] concluded that filtered cell-free medium from irradiated human epithelial cells significantly reduced clonogenic survival and increased the incidence of apoptosis in non-irradiated recipient cells. However, the same research group also demonstrated that cell-to-cell contact through gap junctions had no effect on the ability of

medium from irradiated epithelial cell cultures to reduce the clonogenic survival of non-irradiated cells [46]. Similarly designed studies [166] showed that low doses of high-LET α-particles may induce extra-cellular factors causing excessive cellular transformation, such as sister chromatid exchange (SCE), in unexposed cells. In this experimental work the short-lived SCE-inducing factors were produced in cell-free irradiated culture medium containing serum. The authors concluded that the short-lived transmissible factors could be involved in the production of super-oxide radicals, while the long-lived factors were considered to be cytokines [164]. Mothersill and Seymour suggested [167] that these short-lived radicals may originate from serum components of the culture medium while long-lived messengers might be released by activated donor cells.

It is still unclear which transmissible cytotoxic factors donor cells release into growth medium during radiation exposure. However, several factors and key pathways at the molecular level have been identified [49]. Among them are CycloOXygenase 2 (COX2), Death Receptor 5 (DR5), InterLeukin (IL), Jun N terminal Kinase (JNK), Nitric Oxide (NO) and NO synthase 2 (NO2), Reactive Oxygen Species (ROS), Transforming Growth Factor β (TGβ) and its receptor (TGβR), Tumour Necrosis Factor α (TNF α).

In this study, we assessed the toxicity of ICCM for the end point related to cell survival. The ability of non-irradiated cells to form colonies after exposure to medium born signals released by irradiated cells measures the long-term cell survival. A standard medium transfer colony-forming assay was chosen as it encompasses all types of cellular damage including early apoptosis, mitotic catastrophe (leading to late apoptosis or necrosis), permanent growth arrest followed by necrosis and cell senescence.

Given the variability of the bystander responses reported in the literature in the current work we aimed to determine whether the occurrence and the magnitude of medium-borne signals could be influenced by different radiation doses received by donor cells. We also aimed to investigate whether

cell concentration, from which ICCM was collected, has an impact on its toxicity. It was out of the scope of the current work to identify diffusible cytotoxic components released by directly irradiated cells into the extra-cellular environment.

4.3. Materials and methods.

4.3.1. Microdosimetric model for the emission of medium-borne cell death signals and recipient cell survival probability.

Stewart *et al* [23] proposed a microdosimetry [168] based model that predicts the magnitude of the emission of death signals released by irradiated donor cells into growth media and that evaluates the probability of recipient non-irradiated cells to survive these signals.

It was assumed that death signal emission is proportional to the number of donor cells releasing these signals due to radiation hits. The secretion of death signals was considered to be a dose related stochastic process depending on the amount of energy deposited into the intra and extra cellular environments.

A brief summary and the main assumptions used in the work of Stewart *et al* are as follows*:*

- the expected number of death signals released by all hit cells (σ) is expressed by Equation 4.1:

$$\sigma(D) = N_c \sum_{n=0}^{\infty} \sigma p(\sigma|n)\, p(n|\bar{n}) = N_c \sum_{n=0}^{\infty} p(n|\bar{n}) \sum_{\sigma=0}^{\infty} \sigma p(\sigma|n) \qquad (4.1)$$

- change in the average number of death signals per donor cell ($\Delta\hat{\sigma}$) attributed to the radiation dose received is shown in Equation 4.2:

$$\Delta\hat{\sigma}(D) \equiv \frac{\sigma(D) - \sigma(0)}{N_c} = \left[\sum_{n=0}^{\infty} \sigma_n p\left(n|\bar{n}\right) - \sigma_0 \right] = \left[\sum_{n=1}^{\infty} \sigma_n p\left(n|\bar{n}\right) - \sigma_n (1 - e^{-n}) \right] \qquad (4.2)$$

- the survival fraction (SF) of the recipient cells exposed to ICCM containing σ death signals is expressed by Equation 4.3:

$$SF = \frac{\sum_{q}(1-\eta)^{q}\, p(q|D)}{\sum_{q}(1-\eta)^{q}\, p(q|0)} = \exp\{-\eta[\sigma(D)-\sigma(0)]\} \equiv \exp[-\eta N_c \Delta\hat{\sigma}(D)] \qquad (4.3).$$

The parameters used in these equations and their meanings are defined as in Table 4.1.

Denoted Parameters	Meaning
n	The total number of radiation hits per cell
σ	The number of death signals emitted
$p(\sigma\|n)$	The probability that σ signals will be emitted by cells hit by radiation n times
$p(\sigma\|n)p(n\|\bar{n})$	The probability that cells hit by radiation in average $\bar{n}$ time emit exact σ number of death signals
N_c	The number of the donor cells exposed to radiation
$N_c\sum_{n=0}^{\infty}\sigma p(\sigma\|n)p(n\|\bar{n})$	The expected number of the death signals released by N_c irradiated cells into the ICCM
σ_0	The number of death signals secreted into ICCM by cells not hit by radiation
η	The probability that a medium borne signal reaches a non-irradiated recipient cell and inactivates it
$1-\eta$	The probably of a non-irradiated cell to survive
q	The number of death signals transferred to a recipient cells
$(1-\eta)^q$	The probability of cells to survive after receiving the exact q

	number of death signals
$p(q\|D)$	The distribution of the number of death signals within the culture medium of the recipient cells after donor cells were exposed to D radiation dose

Table 4.1. The parameters used by Stewart et al in the microdosimetry based model predicting cell-killing through medium-borne death signals.

According to equation 4.3, the fraction of non-irradiated recipient cells, surviving transfer into the ICCM, decreases exponentially with increase in the signal intensity $(\Delta\hat{\sigma}(D))$ or the number of irradiated donor cells (N_c).

In the current work we aimed to investigate whether the toxicity of ICCM depends on the absorbed dose received by donor cells and on the cellular concentration of donor cells releasing toxic medium-borne signals due to radiation hits.

4.3.2. Cell culture.

The PC3 human prostate epithelial adenocarcinoma cell line, initially established from a patient' bone marrow metastasis [114] was used in this work. This cell line was kindly provided by Prof Wayne Tilley from the Dame Roma Mitchell Cancer Research Laboratories, University of Adelaide and the Hanson Institute.

The PC3 cell line was used as the donor, from which the ICCM was derived. The same cell line was used as the recipient non-irradiated cells, to which the ICCM was transferred.

These cells were cultured as a monolayer in RPMI 1640 (Sigma, Sigma-Aldrich Co, USA) growth medium, supplemented with 10% foetal calf serum (FCS) (Gibco, USA), 1m*M* L-Glutamine,

1% sodium pyruvate, 1% ascorbate, 1% penicillin/streptomycin at 37^{o}C in a humidified atmosphere containing 5% /CO_2.

4.3.3. Cell irradiation.

Cell irradiation was performed by using a 6 MV x-ray beam produced by a Varian linear accelerator (Varian, Palo Alto, CA,USA) at the Royal Adelaide Hospital Radiation Oncology Department (South Australia).

Irradiation was performed at 100 cm from the beam focal spot with a 20 x 20 cm^2 radiation field size by applying 3 Gy/min clinically used nominal dose rate. The culture flasks were placed on top of 1.5 cm thick solid water build up sheets to achieve electronic equilibrium at the cell surface. To avoid irradiation through air gaps cells were treated posteriorly with the gantry positioned at 180^{o}. To ensure full scatter conditions a custom made wax phantom was applied in conjunction with a 5 cm thick solid water slab to cover the flasks.

Cells were irradiated in T-75 flasks (Corning, USA), containing 15 ml of the culture medium per flask.

- To investigate the toxicity of the ICCM, based *on the dose received by donor cells* 2, 4, 6 and 8 Gy absorbed dose, were delivered to each flask to the cell monolayer surface.
- To investigate the toxicity of the ICCM based on *the donor cells concentration 2 Gy* absorbed dose was delivered to each flask containing different amounts of cells.

The doses, which closely mimic human exposure scenario in radiation therapy, were chosen in this study.

The beam-on-time parameters, so called monitor units (MU), were determined by using LiF100 thermoluminecent chip dosimeters (TLD). TLDs were used to establish the MUs required to give the stated absorbed dose to the cell applying the described radiation setup.

Prior to irradiation the machine radiation output was routinely checked with a Daily QA 3™ device (Sun Nuclear, USA) ensuring that 1 Gy of absorbed dose was delivered under reference conditions. Following irradiation the culture flasks were placed into the incubator immediately.

4.3.4. ICCM derivation.

4.3.4.1. Based on the absorbed dose received by donor cells.

The medium transfer protocol used to derive the ICCM can be found described in detail elsewhere [169]. Briefly, approximately $1.3x10^6$ of PC3 donor cells per T75 flask were plated 24 h prior to irradiation to let the cells adhere to the flasks surface. Then, 15 ml of the fresh cell culture medium was added to each flask just before irradiation.

Absorbed doses of 2, 4, 6 and 8 Gy were delivered to each flask using the linac irradiation setup described above. The control flask, containing the same amount of PC3 donor cells received no irradiation.

The ICCM from donor cells, corresponding to each dose received, was carefully collected 1 h post irradiation and filtered through a 0.22 μm filter to ensure that no cells or other debris were left in the medium.

A 1h incubation for the generation of ICCM was chosen based on published work that demonstrated no change in the recipient cells responses when the medium was harvested over the period from 30 min up to 24 h [164].

4.3.4.2. Based on the cellular concentration of donor cells exposed to the same dose.

Donor cells corresponding to the approximate cellular concentrations of $2x10^2$, $5x10^3$, $5x10^4$, $5x10^5$ and $5x10^6$ per T75 flask were seeded 24 hours before irradiation.

Each flask contained 15 ml of the fresh culture medium and was exposed to an absorbed dose of 2Gy. A T75 flask containing cell-free culture medium was used as the control.

Irradiated cell condition medium from the donor flask, corresponding to each cellular concentration, was carefully harvested 1 h after irradiation and filtered through a 0.22 μm filter to ensure that no cells or other debris were still present in the medium. Cell-free culture medium from the control flask was collected at the same time.

4.3.5. Clonogenic assay.

Determination of cell survival after direct irradiation of cells or exposure to ICCM was performed by the clonogenic assay technique of Puck and Marcus [170].

4.3.5.1. Cellular survival of directly irradiated donor cells.

Following the ICCM collection from the donor flasks exposed to different amounts of radiation dose, cells were washed with Hanks balanced salt solution (HBSS) and harvested from flasks (i.e. released by incubation with trypsin (a 1:1 solution of 0.25% trypsin and 1 m*M* EDTA) for 5 min in 37 ^{0}C at 5% CO_2). Cells were resuspended in HBSS, containing 5% FCS and centrifuged (4000 rpm, 4 ^{0}C). Following detachment, the cells were washed once. Cells' pellets were resuspended in a fresh growth medium, and syringed carefully to produce a single cell suspension.

The number of viable cells was determined by using trypan blue exclusion. An aliquot of the cell suspension was mixed with trypan blue dye and counted using a hemocytometer.

Cells were plated into 6 well dishes (Corning, USA) containing 2 ml of the fresh culture medium. The plating density was chosen based on the preliminary investigation of the PC3 cloning

efficiency and the radiation doses received. The seeding densities varied from 200 cells/well for the control group up to 3300 cells/well for the flask receiving 8 Gy.

4.3.5.2. Survival of the recipient cells receiving the ICCM derived from the donor cells exposed to varying radiation doses.

Two hundred non-irradiated PC3 cells, called the recipient cells, were plated in each well into 6 well dishes (Corning, USA) at the same time as the donor cells and incubated in 37 ^{0}C at 5% CO_2 for 24 hours.

Each well contained 2 ml of the culture media which was replaced with 2 ml of the ICCM collected from the donor cells received 2, 4, 6 and 8 Gy of the absorbed dose or non-irradiated.

4.3.5.3. Survival of the recipient cells exposed to the ICCM derived from various concentrations of the donor cells.

Two hundred PC3 recipient cells were plated per well into 6 well dishes (Corning, USA) at the same time as the donor cells and incubated in 37 ^{0}C at 5% CO_2 for 24 hours.

Each well received 2 ml of the ICCM derived from the $2x10^2$, $5x10^3$, $5x10^4$, $5x10^5$ and $5x10^6$ of donor cells exposed to 2 Gy absorbed dose. The control group received cell free medium exposed to 2 Gy.

For both experiments all culture dishes were incubated for two weeks according to the previously estimated PC3 doubling time in order to mature into colonies containing more than 50 cells per colony and to exclude cells which have a limited growth potential.

Colonies were then fixed with methanol, stained with crystal violet and counted using a stereomicroscope.

The colony forming ability of cells receiving either direct radiation damage or being exposed indirectly to the ICCM was based on the counted number of colonies. Cell survival fractions for each treatment were estimated by applying the following ratio [120]:

$$SF = \frac{colonies\ counted}{cells\ plated} \times CFE \qquad (4.4)$$

Where CFE is the colony forming efficiency estimated from the non-irradiated control group by applying the following ratio:

$$CFE = \frac{colonies\ formed}{cells\ plated} \times 100\% \qquad (4.5)$$

The number of surviving colonies in control plates, scored for each experimental setup, was considered as 100 %. The colonies estimated from the treated samples were normalised to the controls. In each experiment cell survival was estimated by using sixfold sampling.

4.3.6. Statistical analysis.

Dose response of cell survival data measured from six fold samplings was expressed as means ± SEM. The level of significance between treated and control groups was analysed in Graph Pad Prism 5 software **(**GraphPad Software, Inc., CA, USA) with one way ANOVA analysis of variances, the test which determines how a response was affected by one factor, followed by Tukey's Multiple

Comparison Test. This statistical approach is different from a regular t test as it considers the scatter of all groups, giving the test more power to detect differences. Furthermore multiple comparisons allow evaluation of the significance level considering the entire family of comparisons instead of each comparison individually. P values of less than 0.05 were considered to be statistically significant. (In Graph Pad Prism 5 software the level of statistical significance can be chosen between 0.05, 0.01, or 0.001, which graphically presented as *, ** and *** symbols respectively). Cell survival curves were fitted in the Graph Pad Prism 5 software by applying non-linear regression one phase decay model.

4.4. Results and discussion.

The experiment reported here was designed to investigate whether survival of non-irradiated cells exposed to the ICCM depends on the amount of radiation absorbed by donor cells from which the medium was harvested. The toxicity of the ICCM was also analysed based on the concentration of donor cells. The results and discussion are presented in the following sections.

4.4.1. Survival of cells received the ICCM derived from donor cells exposed to varying radiation doses.

There were significant ($P < 0.001$) decreases in cellular survival of the recipient cells that received ICCM derived from the donor cells exposed to 2, 4, 6 and 8 Gy absorbed radiation doses compared to the control group (see Figure4.1).

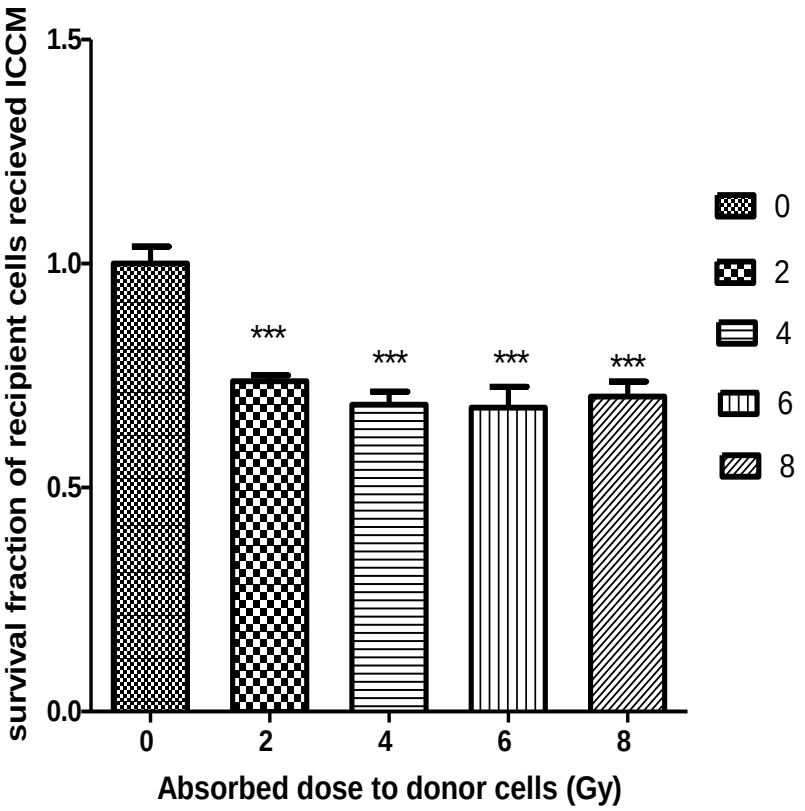

Figure 4.1. The survival fraction of non-irradiated PC 3 cell after receiving ICCM from donor cells exposed to 0, 2, 4, 6 and 8 Gy of absorbed dose.

In the current work it was observed that exposure of PC3 recipient cells to the ICCM, containing the currently unknown medium-borne signals, caused changes in colony forming abilities in this cell line.

However, it can be noted that the media harvested from each irradiated group of the donor cells was approximately equally toxic regardless of the radiation dose. According to Table 4.2 there were not any significant dose-dependant differences observed between survival of cells exposed to ICCM treated with 2 Gy, 4 Gy, 6 Gy or 8 Gy radiation doses.

Test No.	Comparison Test	Mean Diff. Survival	Significant? P < 0.05?	Summary
1	0 Gy vs 2 Gy	26.2%	Yes	***
2	0 Gy vs 4 Gy	31.5%	Yes	***
3	0 Gy vs 6 Gy	32.2%	Yes	***
4	0 Gy vs 8 Gy	29.7%	Yes	***
5	2 Gy vs 4 Gy	5.3%	No	ns
6	2 Gy vs 6 Gy	5.9%	No	ns
7	2 Gy vs 8 Gy	3.5%	No	ns
8	4 Gy vs 6 Gy	0.7%	No	ns
9	4 Gy vs 8 Gy	-1.8%	No	ns
10	6 Gy vs 8 Gy	-2.5%	No	ns

Table 4.2. One-way analysis of variances with Post ANOVA Tukey's Multiple comparison tests of the PC3 cell survival after receiving ICCM exposed to 0, 2, 4, 6 and 8 Gy radiation doses.

This was in contrast to the survival of cells that received direct radiation damage (see Figure 4.2), which indicates rapid, *dose-dependant* decrease in SF compared to the cells receiving indirect radiation damage.

The difference in cell survival patterns between direct and indirect radiation damages suggests that perhaps donor cells, activated by relatively high radiation doses, released a similar amount of transmissible cytotoxic factor(s) into the cell growing medium regardless of the doses received.

It appears that initially emitted death signals may increase with increasing number of hits received by donor cells. However, it saturates when the number of hits per cell reaches a certain value [171]. There was rapid drop-off (26.6%) in the cell survival when the recipient cells received ICCM exposed to 2 Gy dose. Nevertheless, after this dose point the fractions of surviving cells exposed to ICCM treated with 4, 6 or 8 Gy dose remained stable (31.5%, 32.2% and 29.7%).

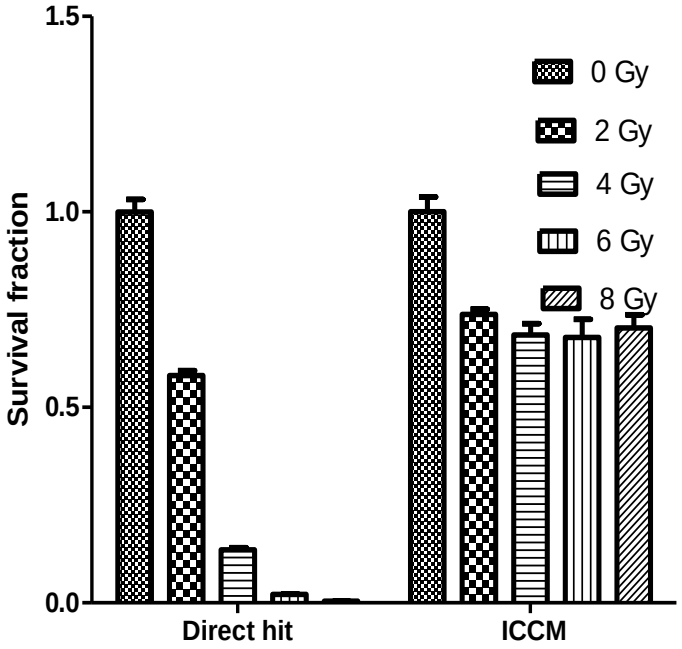

Figure 4.2. The survival fraction PC3 cells after exposure to direct hit radiation or ICCM.

This indicates that the emission of medium-born signals becomes saturated at a certain level of radiation exposure, which was below 2 Gy.

Our observation is in good agreement with the experimental work of Seymour and Mothersill [169] who observed a relatively constant effect of the ICCM on cell survival in human epithelial cell lines. However, they concluded that the magnitude of the effect appeared to saturate at doses in the range of 0.03-0.05 Gy. Their study reported that for ICCM exposed to doses higher than 0.5 Gy the

clonogenic cell deaths observed were either 'a result of a dose-dependent non-bystander effect or a dose-independent bystander effect'.

Another research group [172] observed no significant difference in ICCM toxicity generated by cells irradiated with either 0.5 Gy or 5 Gy in the experimental work with human keratinocytes. This indicates the saturation of the medium-born death signals at doses below 0.5 Gy in that experiment. Nevertheless, Maguire at el [43] observed in human keratinocytes a significant dose-dependent increase in mitochondrial mass per cell exposed to ICCM irradiated with 5 mGy and 0.5 Gy doses. The reported results suggest that the toxicity of ICCM was dose dependent but only at the low level of radiation exposure below 0.5 Gy.

In the current experimental work we were able to observe significant decrease in the cell survival exposed to the ICCM. The cell survival curve fitting ($\chi 2=0.99$) confirmed an exponential relationship between the fraction of the cell surviving exposure to ICCM and the absorbed doses received by donor cells (see Figure 4.3).

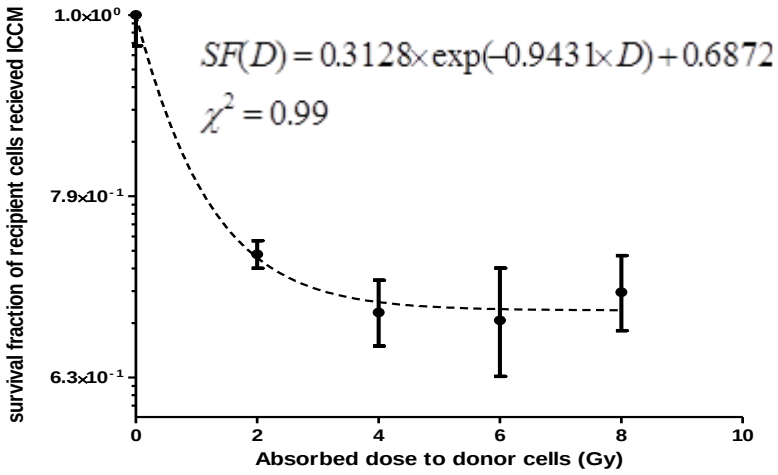

Figure 4.3. The exponential fit according to Stewart et al of PC3 cells survival fraction after exposure to ICCM from donor cells receiving a direct hit of radiation ranging from an absorbed doses of 2 to 8 Gy.

However we did not observe dose dependant variation in ICCM toxicity above 2 Gy, which was most likely a result of the high level of the radiation exposure used. The secretion of medium-borne signals by PC3 donor cells into the extracellular environment may increase at low doses. However it reached a plateau and became saturated after a currently unknown absorbed dose.

To identify a dose threshold and to further analyse a dose-dependent signal emission of the PC3 cells the low dose region (below 1 Gy) needs to be investigated.

4.4.2. Survival of the recipient cells receiving ICCM derived from varying concentrations of the donor cells exposed to 2 Gy.

In this experiment it was investigated whether the amount of donor cells releasing transmissible cytotoxic factor(s) may affect the toxicity of ICCM.

A significant decrease in cell survival, according to the Table 3, was found in the recipient cells exposed to the ICCM derived from donor cells of the different concentrations ($2x10^2$, $5x10^3$, $5x10^4$, $5x10^5$, $1.3x10^6$ and $6x10^6$) exposed to a 2 Gy radiation dose compared to the control that received cell-free non-irradiated media (see Figure 4.4).

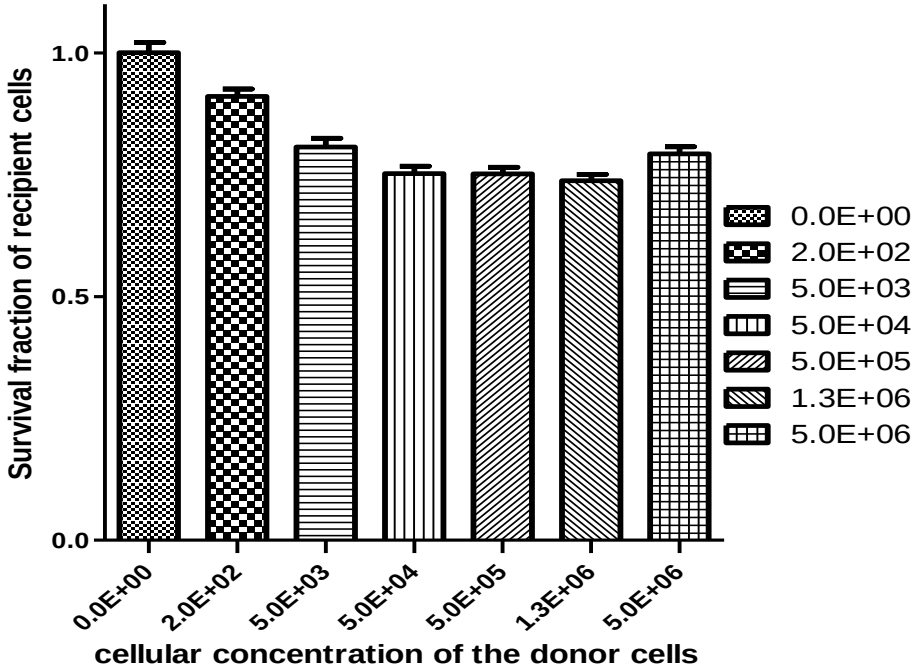

Figure 4.4. The survival fraction of recipient PC 3 cells after receiving ICCM exposed to 2 Gy of an absorbed dose derived from donor cells of different cellular concentrations (0, 2.0E+02, 5.0E+03, 5.0E+04, 5.0E+05, 1.3E+06, 5.0E+06) cells per 15mL .The control group was exposed to cell-free irradiated media received 2 Gy of an absorbed dose.

These differences in SFs, presented in the 1 – 6 comparison tests in Table 4.3 may be attributed to either the amount of radiation received by the ICCM or to the cellular concentration of donor cells releasing medium-borne signals.

However, further evaluation of the rows 7 – 11 presented in the Table 4.3 revealed significant differences ($P<0.001$) between cells surviving the exposure to the ICCM, derived from 200 donor cells exposed to 2 Gy, and the rest of donor cell concentrations (12 – 21 comparison tests) receiving the same dose. These results exclude the radiation dependence component in assessing ICCM toxicity and demonstrate the variation among the different cellular concentrations releasing medium-borne signals.

Test No.	Comparison Test	Mean Diff. Survival	Significant? P < 0.05?	Summary
1	0 vs 200	9.0%	Yes	**
2	0 vs 5000	19.3%	Yes	***
3	0 vs 50000	24.8%	Yes	***
4	0 vs 500000	24.9%	Yes	***
5	0 vs 1300000	26.3%	Yes	***
6	0 vs 5000000	20.7%	Yes	***
7	200 vs 5000	10.4%	Yes	**
8	200 vs 50000	15.8%	Yes	***
9	200 vs 500000	15.9%	Yes	***
10	200 vs 1300000	17.3%	Yes	***
11	200 vs 5000000	11.8%	Yes	***
12	5000 vs 50000	5.5%	No	ns
13	5000 vs 500000	5.6%	No	ns
14	5000 vs 1300000	7.0%	No	ns
15	5000 vs 5000000	1.4%	No	ns
16	50000 vs 500000	0.1%	No	ns
17	50000 vs 1300000	1.5%	No	ns
18	50000 vs 5000000	4.1%	No	ns
19	500000 vs 1300000	1.4%	No	ns
20	500000 vs 5000000	4.1%	No	ns
21	1300000 vs 5000000	5.5%	No	ns

Table 4.3. One-way analysis of variances with Post ANOVA Tukey's Multiple Comparison Test of the PC3 recipient cells survival exposed to the ICCM derived from varying concentrations of the donor cells exposed to 2 Gy radiation dose.

Thus, it was shown that SF of non-irradiated cells was significantly reduced after receiving ICCM harvested from 200 donor cells. However after the 5000 cell concentration point the toxicity of ICCM reaches a plateau and becomes saturated.

This indicates that for PC3 cell line under the current experimental conditions, there was an observable decrease in SF of non-irradiated cells exposed to ICCM depending on the cellular concentration.

Observed literature revealed no data of similar design to investigate ICCM toxicity based on the cellular concentration of donor cells. However Ryan *et al* [173] investigated the intensity of medium-borne signals based on the dilution of ICCM derived from the six human cell lines which were transferred to the common reporter HPV-G recipient cells. Their study revealed that ICCM from cells of four cell lines induced a bystander effect in HPV-G reporter cells, confirming that signal production was an essential factor. This experimental work also confirmed that the intensity of ICCM varied with dilutions.

Ryan *et al* suggested that cell survival would decrease linearly until a plateau was reached and the bystander effect was abolished. It was considered that the effect of ICCM from the different cell concentrations reached a plateau at different dilutions. It was also suggested that the intensity of the emission of medium-borne signals was correlated with the cell line' radiosensitivity.

Our cell survival curve fitting ($\chi 2$ =0.9) revealed an exponential relationship between the fraction of cells surviving the exposure to the ICCM and the cellular concentration of donor cells releasing medium-born signals (see Figure 4.5). It appeared that the toxicity of ICCM increased exponentially with donor cell concentration, reached plateau and become saturated after the 5000 cells concentration point.

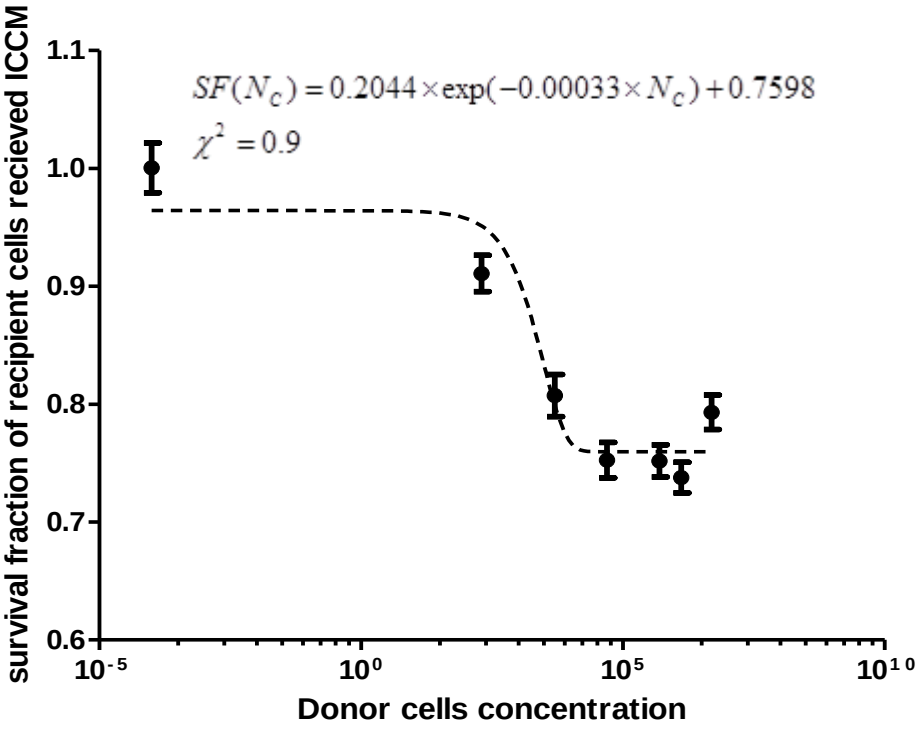

Figure 4.5. The exponential fit of PC3 cells survival fraction after exposure to the ICCM derived from 0, $2x10^2$, $5x10^3$, $5x10^4$, $5x10^5$, $1.3x10^6$ and $6x10^6$ donor cells per 15mL exposed to an absorbed dose of 2 Gy.

In the current experimental work it was investigated whether the fraction of non-irradiated recipient cells, surviving the exposure to ICCM, was dependent on the cellular concentration and the absorbed dose received by donor cells.

It was assumed that the SF of the recipient cells exposed to ICCM exponentially decreases (as expressed in equation 6) with increase in the signal intensity *($\Delta\hat{\sigma}(D)$)* and the number of irradiated donor cells (N_c) emitting medium-borne death signals.

$$SF \equiv \exp[-\eta N_c \Delta\hat{\sigma}(D)] \tag{4.6}$$

$$SF(D) \equiv A1 \times \exp[-B1 \times f(D)] + C1 \tag{4.7}$$

$$SF(N_C) \equiv A2 \times \exp[-B2 \times f(N_C)] + C2 \tag{4.8}$$

The data measured in the current work were fitted with equations (4.7) and (4.8).
These equations provide a reasonable agreement with Equation (4.6) proposed by Stewart.

Thus, an equation (4.7) indicates the exponential decrease in recipient cell survival as a function of the doses absorbed by donor cells. Additionally, equation (4.8) demonstrates that the intensity of the ICCM, affecting recipient cells, was dependent on the concentration of donor cells releasing toxic medium-born signals as a result of radiation hit.

It was assumed that the constants presented in the equations (4.7) and (4.8) depended on experimental setup, cell growing and treatment conditions.

Similarly to direct radiation damage intercellular-mediated effects may depend on many factors related to biological and exposure conditions presented in a certain experimental reference frame.
Thus it has been demonstrated that the magnitude of the intercellular-mediated effects was dependent on the serotonin level present in FCS [174].

The choice of relevant experimental times in medium transfer experiments was shown [175] to be crucial not only for identifying the effects but also for detecting them. Still, the previously conducted study by Mothersill *et al* [164] demonstrated insignificant variations in non-irradiated cell responsiveness to ICCM derived either 30 minutes or 24 hours after radiation exposure.

Additionally, it was shown that radiation induced intercellular-mediated damage was cell line dependant [164, 176]. However, another research group demonstrated that it did not correlate with cellular intrinsic radio sensitivity [177].

Dose and dose rate dependence of the medium transfer effect is another controversial point. Several studies suggest that dose [169, 172, 177] and dose rate [178] are uncorrelated in their medium transfer experiments. However another research group [43] confirmed the opposite dose dependant relationship.

4.5. Conclusion.

The microdosimetry based stochastic model of Stewart *et al* predicts the magnitude of the emission of death signals released by irradiated donor cells into growing media and evaluates the probability of recipient non-irradiated cells to survive these signals. It provides a more systematic approach to analyse dose dependency and signal potency in medium transfer experiments. In the current work we made an attempt to validate the proposed formalism of Stewart *et al* which predicts an exponential decrease in recipient cell survival based on the energy deposition to donor cells and the number of donor cells releasing these signals due to radiation hit. It was not the intention of this work to determine the mechanisms underlying the emission of death signals.

Significant reduction in PC3 cell survival after receiving ICCM was observed. Data fitting revealed an exponential decrease in recipient cell survival; however it was not possible to identify a dose threshold due to the saturation of the effect at a currently unknown dose. This can be attributed to either saturation in signal generation due to limited signal potency or saturation in recipient cell responses. It appeared that death signal emission may increase with increasing number of radiation hits to a certain target and with increasing number of targets able to emit death signals. However, the effect saturates when it reaches a specific value in a number of hits or in an amount of critical targets. Additional data, preferably performed in a more consistent experimental reference frame, are needed to determine the origin of the critical target(s). In this study we confirm that the stochastic microdosimetry based model of Stewart *et al* captures the main trends observed in low-LET medium transfer effects.

Chapter 5.0

Conclusions and future work.

There is a potential for RIBEs to be either beneficial or detrimental in application to radiotherapy treatment. Recent developments in the delivery of external beam radiation therapy including intensity modulated radiotherapy (IMRT), helical tomotherapy and heavy particle ion therapy indicate the necessity for further investigation of RIBEs. There is some probability that non-targeted effects of ionising radiation can modulate therapeutic outcome under conditions where significant variation in steepness of dose gradient, dose rate and increased irradiation delivery time are present.

Research described in this thesis aimed to investigate impact of non-targeted effects of ionising radiation to the application of radiotherapy. For this reason, a series of *in vitro* radiobiological experiments were performed with PC3 human prostate adenocarcinoma cell line. Experiments were undertaken to test whether radiation induced additional killing occurs and has an impact on a single fraction radiotherapy treatment outcomes such as cell survival.

From the literature review undertaken, experiments were designed to test the following hypothesis;

1. That intercellular signalling and induced toxicity of irradiated cell condition media during radiotherapy treatment may contribute substantially to reduce cell survival in regions of a 20% under-dosed and;

2. That the magnitude of medium-borne signals (affecting non-targeted cells) depends on the radiation doses received by donor cells and donor cell concentration.

It was found that for the cell line investigated under identical growth and treatment conditions, significantly different survival was observed for cells under-dosed by 20%, that were either in

physical contact with the recipient cells receiving 100% of the prescribed dose (2 Gy) or irradiated separately. It is presumed the variation in survival was due to the cells receiving a20% under-dose being in cellular contact during and shortly after irradiation with cells that received 100% of the prescribed dose.

Significant reduction in the survival of PC3 cell after receiving ICCM was also observed. Data fitting revealed an exponential decrease in recipient cell survival. The current experiment was not able to identify the associated dose threshold for the reduction in survival from ICCM due to the saturation of the effect at the doses investigated. Death signal emission may increase with increasing number of radiation hits to a certain target and with an increase in the number of targets that are able to emit death signals. However, the affect saturates when it reaches a specific value in a number of hits or in an amount of critical targets available.

The observed statistically significant decrease in cell survival in regions of a 20% under-dose in communicating PC3 cells compared to non-communicating cells does not however suggest that at present one can clinically ignore a dose cold spot in a treatment plan and rely on intercellular signalling, which may or may not inactivate an unknown amount of clonogenes. It has been postulated [179] that neither bystander effects nor radiation-induced genomic instability would be able to contribute substantially to achieving local tumour control of the primary cancer. Woodard *et al* suggests that a local recurrence after curative radiotherapy arises from a single or a few tumour 'stem' cells which may, by chance, have not been lethally hit by radiation doses delivered and retained their unlimited proliferative potential [180]. However, the progeny of these surviving tumour 'stem' cells are likely to suffer from radiation-induced genomic instability, which results in the persistent appearance of non-stem cells, i.e. a reduced probability of self-maintenance. This leads to a slower growth rate of the recurrent tumour, a reduced stem cell fraction and, as a consequence, an increased radiosensitivity of the recurrent tumour [179].

In external beam radiotherapy, few radiobiologists would doubt that each tumour 'stem' cell has to be destroyed either directly, by hitting a crucial target in its nucleus or, in some cases, by vascular effects such as those from hyperthermia or photodynamic therapy or by additive cytotoxicity in combined radio-chemotherapy. It has been proposed [15] that in situations of inhomogeneously irradiated tumour cell populations, which is quite common in the therapeutic application of unsealed radionuclides that either delivered directly or attached to some tumour-specific carriers, the negative effects of inhomogeneity can be corrected by bystander effects. It is assumed that cells that are lethally affected would send death signals to neighbouring, bystanding cells that are not directly affected. Freely diffusible toxic bystander signals could potentially overcome the inefficiency of tumour control due to non-uniform distribution of radiation dose.

Elucidation of the pathways involved in RIBE generation by radionuclides could indicate ways of manipulating RIBE production to reduce toxicity of normal tissues inadvertently irradiated during the course of a targeted radiotherapy. Careful choice of radionuclides and dose administered in clinical scenarios for targeted radionuclide therapy of tumours which naturally accumulate targeted radionuclides or have been genetically manipulated to do so, will allow factors such as inefficient gene transfer and heterogeneous uptake to be compensated for, thus optimising the cell kill potential of this therapeutic scheme.

Whatever the mechanism, RIBE could be important not only in relation to radiation protection and safety but also with respect to the therapeutic use of ionizing radiation. From a practical perspective, the identification of RIBE factors will stimulate the design of strategies to maximize damage to tumour cells while minimizing damage to normal cells.

One of the main questions which still remain to be answered is the nature of signaling and transducing molecules. Much concerted research has failed yet to exactly identify these, presuming they may be complex or that multiple steps and pathways may be involved. The identification of

these bystander factors will stimulate the design of radiotherapy strategies to maximize damage to tumor cells while minimizing damage to normal cells. It can provide a whole new range of targets for novel radiotherapy techniques, including drugs and radiopharmaceuticals. However, from the practical point of view it is not necessary to understand why, what, or how to exploit bystander effects for therapy.

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