INVESTIGATION OF X-RAY RADIATION-INDUCED BYSTANDER EFFECTS IN CANCEROUS MCF-7 AND NORMAL MCF-10A BREAST CELL LINES

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by

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LIST OF SYMBOLS

α	Alpha
β	Beta
ºC	Degree celsius
cm	Centimetre
cm^2	Square centimetre
CO2	Carbon dioxide
γ	Gamma
G	Gram
Gy	Gray
HO•	Hydroxyl
Mg	Milligram
µl	Microliter
ml	Millimeter
mMol/L	Milli mol per liter
mM	Millimolar
µMol/L	Micro Mol per Litre
MN	Micronuclei
MV	Megavoltage
ND_w	Absorbed dose in water
$O2^-$	Superoxide
$ONOO^-$	Peroxynitrite
pH	Neutralization buffer
RO2•	Alkoxy
R^2	Linear relationship value

LIST OF ABBREVIATIONS

AE	Abscopal effect
AES	Alkaline electrophoresis solution
ANOVA	One-way analysis of variance
ARS	Acute radiation syndrome
ATM	Ataxia-telangiectasia mutated
BRCA1	Breast cancer gene 1
BSC	Biosafety cabinet
BED	Biologically effective dose
BER	Base excision repair
C	Carbon
CASP	Comet assay software project
Cd^{2+}	Cadmium
cfCh	Cell-free chromatin particles
COX-2	Cyclooxygenase-2
CNMC	Centro Nacional de Metrología de Cuba
dH2O	Distilled water
Dmax	Depth of maximum dose
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DDR	DNA damage response
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	Double strand breaks

DSIC	Distant cell signalling intercellular communication
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
FBS	Fetal Bovine Serum
G-CSF	Granulocyte colony-stimulating factor
GJIC	Gap junction intercellular communication
HCL	Hydrochloric acid
HD	High-dose
HDR	High Dose Rate
HEPA	High-Efficiency Particulate air
HR	Homologous recombination
HRS	Hyper radiosensitivity
H_2O_2	Hydrogen peroxide
H_2AX	histone family member X
ICCM	Irradiated-cell conditioned medium
IL-1β	Interleukin
IL-8	Interleukin-8
IL-6	Interleukin-6
IR	Ionizing radiation
IRR	Induced radioresistance
Ku70	Ku heterodimer protein
LD	Low-dose
LET	Linear energy transfer
LMA	Low melting agarose
MR	Mismatch repair

μ-FT-IR	Fourier transform infrared microspectroscopy
MAPK	Mitogen-activated protein kinase
MC	Mitotic catastrophe
NACL	Sodium chloride
NAOH	Sodium hodroxide
NER	Nucleotide excision repair
NF-kB	Nuclear factor-κB
NHEJ	Non-homologous end joining
NMPA	Normal melting point agarose
NO	Nitric oxide
NTE	Non-target effect
OTM	Olive Tail Moment
PBS	Phosphate buffered Saline
PEITC	Phenethyl isothiocyanate
PET	Positron emission tomography
Rad51	Homologous recombination protein
RBE	Relative biological effectiveness
RIBE	Radiation-induced bystander effects
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions Per Minute
RT	Radiotherapy
S	Senescence
SD	standard deviation

SPET	Single photon emission computed tomography
siRNA	Small interfering RNA
SSB	Single strand break
SSD	Source to surface distance
tAIF	Truncated apoptosis inducing factor
TGF-β1	Transforming growth factor-β1
TNF-α	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor
XRCC1	X-ray repair cross-complementing protein 1
Zn^{2+}	zinc
WST-1	(4-[3-4-iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio)- 1,3-benzene disulfonate

KAJIAN KESAN BYSTANDER YANG DIDORONG OLEH SINAR-X TERHADAP GARIS SEL PAYUDARA MCF-7 KANSER DAN GARIS SEL MCF-10A NORMAL

ABSTRAK

Kesan sinaran mengion ke atas sel dan tisu hidup telah dikaji secara meluas. Interaksi sinaran mengion mencetuskan pelbagai tindak balas biologi dalam sel yang disasarkan. Walau bagaimanapun, ia juga boleh mencetuskan kesan terhadap sel yang tidak disasarkan atau kesan bystander yang di induksi oleh sinaran (RIBE). Kajian semasa ini menyiasat tindak balas sel yang disasarkan dan bystander serta impak dos sinaran terhadap RIBE dalam titisan sel kanser MCF-7 dan titisan sel payudara normal MCF-10A manusia. Kajian ini dijalankan menggunakan radioterapi sinar-X dengan sinar foton klinikal 6 MV pada dos yang berbeza (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, dan 5 Gy). Teknik ko-kultur transwell yang diubahsuai dengan inkubasi selama 48 jam digunakan untuk merangsang RIBE. Potensi sitotoksik sinaran secara langsung dan kesan bystander (RIBE) terhadap sel yang disasarkan dan sel bystander yang tidak disinari dinilai menggunakan ujian WST-1. Keputusan menunjukkan bahawa daya maju sel telah direncatkan secara bergantung kepada dos untuk kedua-dua sel kanser dan sel payudara normal dalam tempoh 48 jam. Sel yang disasarkan menunjukkan tindak balas sinaran yang signifikan dalam daya maju sel mereka, sehingga kira-kira 45% selepas inkubasi, manakala sel bystander yang tidak disinari menunjukkan daya maju yang ketara melebihi 77%. Perbandingan ini menekankan kesan berbeza antara pendedahan sinaran secara langsung berbanding kesan bystander. Seterusnya, kajian radiobiologi dijalankan untuk menyiasat kesan sinaran mengion (IR) terhadap morfologi sel dalam titisan sel MCF-7 dan MCF-10A, yang menunjukkan peningkatan

pergantungan dos di dalam dalam apoptosis, nekrosis, dan tapak jalan kematian sel lain, terutamanya pada dos melebihi 3.5 Gy. Perubahan morfologi, termasuk apoptosis dan bencana mitotik, didokumentasikan, dengan dos yang lebih tinggi daripada IR menghasilkan kerosakan DNA yang ketara dan apoptosis sel. Perubahan lipatan relatif di dalam kedua sel sasaran dan sel bystander menunjukkan hubung kait positif dengan peningkatan dos sinaran untuk kdua titisan sel. Kajian ini melibatkan analisis yang teliti untuk mengukur kerosakan DNA di dalam kedua titisan sel menggunakan ujian komet. Hasil menunjukkan peningkatan linear yang signifikan dalam kerosakan DNA bagi sel yang disasarkan dan sel bystander apabila dos sinaran meningkat. Secara khusus, Ujian Komet menunjukkan bahawa kerosakan DNA dalam sel kanser MCF-7 yang disasarkan meningkat daripada *olive tail moment* 0.98 ± 0.5 pada 0 Gy kepada 105.9 ± 1.1 pada 5 Gy. Begitu juga, sel MCF-10A normal yang disasarkan meningkat daripada 4.9 ± 1.09 pada 0 Gy kepada 100.5 ± 1.3 pada 5 Gy. Sel bystander yang tidak disinari juga menunjukkan peningkatan *olive tail moment*, dengan sel MCF-7 meningkat daripada 0.94 ± 0.3 pada 0 Gy kepada 96.1 ± 1.04 pada 5 Gy dan sel MCF-10A meningkat daripada 4.5 ± 0.9 pada 0 Gy kepada 85.1 ± 0.9 pada 5 Gy. Keputusan ini mengesahkan hubungan bergantung dos antara kerosakan DNA yang disebabkan oleh sinaran dan kepekaan selular. Sel kanser MCF-7 menunjukkan radiosensitiviti yang lebih tinggi, dengan penurunan daya maju sehingga 50% untuk sel sasaran dan lebih 33% untuk sel pemerhati pada 5 Gy. Sebagai perbandingan, sel normal MCF-10A menunjukkan penurunan yang lebih kecil: sehingga 40% dalam sel sasaran dan lebih 19% dalam sel pemerhati. Ini mencerminkan perbezaan yang jelas dalam radiosensitiviti antara kedua-dua jenis sel. Penemuan ini menekankan bagaimana jenis sel dan dos sinaran membentuk hasil RIBE. Mereka juga menyerlahkan potensi untuk

mereka bentuk terapi sinaran yang lebih selektif yang meningkatkan penyasaran kanser sambil menyelamatkan tisu yang sihat.

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ABSTRACT

Effects of ionizing radiation on living cells and tissues have been extensively investigated. Ionizing radiation interactions induce various biological responses within the targeted cells. Nevertheless, it might also trigger effects in non-targeted cells or the radiation-induced bystander effect (RIBE). The current study investigates targeted and bystander cell responses and the impact of radiation dosage on RIBE in MCF-7 cancerous and MCF-10A normal human breast cell lines. The studies were conducted utilizing external beam radiotherapy, which involved the application of X-rays with a 6 MV clinical photon beam at different doses (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 Gy). A modified transwell co-culture technique with incubation for 48 hours was employed to induce RIBE. The cytotoxic potential of direct irradiation and bystander effects (RIBE) on targeted and non-irradiated bystander cells was assessed using the WST-1 assay. The results indicated that cell viability was inhibited in a dose-dependent manner for both cancerous and normal breast cells within 48 hours. The targeted cells presented a significant radiation response in their viability, up to approximately 45% after incubation, whereas the non-irradiated bystander cells exhibited a marked viability of over 77%. This comparison underscores the differential impact of direct radiation exposure versus bystander effects. Subsequently, radiobiological studies were undertaken to investigate the effects of ionizing radiation (IR) on cell morphology in MCF-7 and MCF-10A cell lines, revealing dose-dependent increases in apoptosis, necrosis, and other cell death pathways, particularly at doses exceeding

3.5 Gy. Morphological alterations, including apoptosis and mitotic catastrophe, were documented, with higher doses of IR resulting in substantial DNA damage and cellular apoptosis. The relative fold change in both target and bystander cells positively correlated with the increasing radiation dosage for both cell lines. The study involved conducting a rigorous analysis to quantify DNA damage in both cell lines using the comet assay. The results revealed a significant linear increase in DNA damage for targeted and bystander cells as radiation doses increased. Specifically, the Comet Assay demonstrated that the DNA damage in targeted MCF-7 cancer cells increased from an olive tail moment of 0.98 ± 0.5 at 0 Gy to 105.9 ± 1.1 at 5 Gy. Similarly, MCF-10A targeted normal cells from 4.9 ± 1.09 at 0 Gy to 100.5 ± 1.3 at 5 Gy. Non-irradiated bystander cells also exhibited an increase in olive tail moment, with MCF-7 cells rising from 0.94 ± 0.3 at 0 Gy to 96.1 ± 1.04 at 5 Gy and MCF-10A cells increasing from 4.5 ± 0.9 at 0 Gy to 85.1 ± 0.9 at 5 Gy. These results confirm a dose-dependent relationship between radiation-induced DNA damage and cellular sensitivity. MCF-7 cancer cells showed higher radiosensitivity, with up to a 50% drop in viability for targeted cells and over 33% for bystander cells at 5 Gy. In comparison, MCF-10A normal cells showed a smaller decrease: up to 40% in targeted cells and over 19% in bystander cells. This reflects clear differences in radiosensitivity between the two cell types. The findings emphasize how cell type and radiation dose shape RIBE outcomes. They also highlight the potential to design more selective radiation therapies that improve cancer targeting while sparing healthy tissue.

CHAPTER 1

INTRODUCTION

1.1 Overview

The discovery of X-rays by Wilhelm Röntgen in 1895 marked a revolutionary advancement in medical science, propelling the field of radiology to the forefront of medical diagnostics and treatment (Busch U., 2017). Over the decades, X-rays and other ionizing radiation have become foundational in diagnosing various conditions and the targeted treatment of cancer, fundamentally altering the oncology landscape (Hall, 2000; Sproull et al., 2023). Radiotherapy (RT), also known as radiotherapy, is a cornerstone modality in the treatment of cancer (Amorim et al., 2023; Feiock et al., 2016). RT offers several advantages over other cancer treatment modalities. These advantages include potentially improved survival rates, enhanced local tumor control, and favorable profiles of both quality of life and treatment-related toxicity compared to alternative therapies (Baskar et al., 2012). Despite their widespread application and the undeniable benefits, they offer in medical science, ionizing radiation, such as X-rays, carries inherent risks due to their potential to cause cellular damage, posing a carcinogenic threat to living organisms (Ashish Chaturvedi & Vinod Jain, 2019; Pethe et al., 2022; Swanson et al., 2004).

Radiotherapy (RT) primarily eliminates cancer cells by inducing DNA damage, either directly through ionisation or indirectly via the generation of free radicals (Baskar et al., 2012; Mavragani et al., 2019; Sia et al., 2020). However, growing evidence shows that ionizing radiation also affects neighboring non-irradiated cells through intercellular signaling, a phenomenon known as the radiation-induced bystander effect (RIBE). In this process, irradiated cells release signaling molecules

such as reactive oxygen species (ROS), nitric oxide (NO), and cytokines, which can trigger gene mutations, altered gene expression, apoptosis, and DNA damage in nearby bystander cells (Azzam et al., 2001; Chen et al., 2020; Lyng et al., 2002; Nagasawa & Little, 1999). RIBE challenges the traditional view of radiation affecting only directly targeted cells, highlighting the complexity of radiation biology and emphasizing the need to consider both direct and indirect effects in optimizing radiotherapy outcomes (Mitchel, 2004; Pouget et al., 2018; Tang et al., 2023). However, elucidating the precise mechanisms underlying the bystander effect remains an ongoing area of investigation, with research exploring how the magnitude of the effect varies depending on the radiation dose, cell type, and other factors (Buonanno et al., 2023; Mitchel, 2004).

The radiation-induced bystander effect (RIBE) occurs when non-irradiated cells exhibit genetic or functional alterations as a result of signals released from neighboring irradiated cells. These effects such as gene mutation, altered gene expression, apoptosis, and DNA damage are mediated by signaling molecules including reactive oxygen species (ROS), nitric oxide (NO), and cytokines (Azzam et al., 2001; Chen et al., 2020; Lyng et al., 2002; Nagasawa & Little, 1999). By extending radiation damage beyond directly exposed cells, RIBE challenges the classical target theory and highlights the need to consider both direct and off-target effects in radiotherapy (Pouget et al., 2018). While the cytotoxic impact of X-rays on targeted cells is well established, increasing evidence shows that bystander responses also produce complex biological consequences, underscoring the importance of intercellular communication in shaping radiation outcomes (Buonanno et al., 2023; Mitchel, 2004).

Addressing the bystander effect requires a multifaceted approach. This approach involves research to uncover its mechanisms, strategies to mitigate its impact, and incorporating this knowledge into clinical practice. Such a comprehensive approach aims to advance the effectiveness and safety of radiotherapy, a critical tool in the fight against cancer.

The advancement in radiobiology, particularly concerning 6 MV X-ray radiation in clinical oncology, signifies a significant leap forward in our comprehension and application of ionizing radiation for cancer treatment. This progress is underscored by research elucidating the relative biological effectiveness (RBE) of 6 MV X-ray radiation with a range of doses (0–5 Gy), highlighting its comparability and efficacy in precisely targeting cancer cells (Howard et al., 2017). However, a crucial consideration in the clinical application of 6-MV X-rays remains a clear understanding of the impact of radiation doses and the dose-response relationship. This understanding is essential for achieving optimal biological damage to cancer cells while minimizing harm to surrounding healthy cells (Nabikhani et al., 2023; Sjostedt & Bezak, 2010). Generally, if the extent of damage caused by RIBE in tumor cells exceeds that in normal cells, it may benefit to augment the therapeutic ratio (Soleymanifard & Bahreyni, 2012a). The current study aimed to assess the cellular responses of bystander and targeted cells during irradiation with a clinical 6 MV X-ray radiation. RIBE responses were investigated between normal and cancerous cells after incubation with irradiated cells in co-cultured medium.

1.2 Problem statement

The primary concern in radiotherapy is the occurrence of biological responses in non-targeted regions, such as radiation-induced bystander effects (RIBE) (Daguenet

et al., 2020). RIBE can contribute to radiation-induced carcinogenesis through several mechanisms, including the induction of secondary cancers, genomic instability, and delayed or immediate mutations (Mothersill & Seymour, 2003). These effects form the mechanistic basis for estimating RIBE risks in radiotherapy. Previous studies have shown that the long-term consequences of off-target radiation effects depend strongly on both dose and radiation quality, particularly in relation to oxidative metabolism disturbances (Buonanno et al., 2011).

Although the two main pathways and molecular mechanisms of RIBE have been studied in detail, there is still limited guidance for clinical application (Wang, 2018; Tang et al., 2023). A major barrier is the incomplete understanding of how different variables interact in realistic biological systems. Questions remain about how the quality and dose of radiation influence bystander signals, and whether these effects vary by cell type (Tang et al., 2023). Multiple biological factors—such as the identity of directly irradiated cells, the characteristics of nearby bystander cells, and the composition of the intercellular environment—likely shape these responses. In addition, physical parameters in radiotherapy, including dose, dose rate, radiation type, and time after exposure, play a crucial role in determining biological effectiveness and advancing treatment models (Daguenet et al., 2020).

To date, numerous studies have applied different concepts of dose–response relationships and examined bystander signals in various cell types (Schettino et al., 2005; Seymour & Mothersill, 2000; Soleymanifard et al., 2016; Yang et al., 2015a). Some have found, for example, that dose–response relationships are largely determined by the type of bystander cell (Soleymanifard et al., 2013). However, the precise link between RIBE magnitude and dose remains unclear (Tang et al., 2023).

This research aims to bridge the critical gap in understanding how dose and cell type influence both targeted and bystander responses in radiotherapy knowledge essential for integrating RIBE considerations into clinical strategies. Unlike previous studies that focused on a single cell type or isolated bystander effects, this study simultaneously compares normal (MCF-10A) and cancerous (MCF-7) breast cells, which differ in radiation sensitivity due to their distinct biological mechanisms: MCF-7 cells resist radiation-induced apoptosis due to impaired intrinsic death pathways (Jänicke et al., 2001), while MCF-10A cells are more sensitive and possess stronger antioxidant defenses (Giallourou et al., 2019). These differences make them ideal for investigating dose-dependent direct and bystander effects. The study also employs a novel integrated methodology combining a transwell co-culture system with clinically relevant graded X-ray doses, enabling a comprehensive, multi-level biological analysis of RIBE with direct biological implications.

Ultimately, this work contributes to improving radiotherapy strategies by refining our understanding of the dose–response relationship for both target and bystander cells, aiming to reduce RIBE risks through selective targeting of cancer cells while better protecting normal, healthy tissue.

1.3 Research objectives

General Objective:

To investigate RIBE response and radiation impact on targeted and bystander cells of MCF-7 and MCF-10A cell lines at different radiation doses.

Specific objectives:

This study aims to achieve the general objective through the following specific objectives:

i) To evaluate the viability of targeted and bystander cells in both the MCF-7 and MCF-10A cell lines using the WST-1 assay.

ii) To analyze and compare morphological changes associated with cell death pathways in targeted and bystander cells of MCF-7 and MCF-10A cell lines using microscopy and image analysis techniques.

iii) To investigate the extent of cellular DNA damage in targeted and bystander cells for MCF-7 and MCF-10A cell lines using the Alkaline Comet Assay.

iv) To assess the influence of cell type and radiation dose on RIBE by evaluating dose-dependent radiotoxicity through cell viability measurements in targeted and bystander MCF-7 and MCF-10A cells.

1.4 Scope of the study

In this in vitro study, the primary focus of RIBE stimulation lies in the co-culture transwell cell insert technique. This technique involves irradiated cells and their culture medium as the primary mediators for inducing the bystander effect in non-targeted cells. This research aims to assess the RIBE response between normal and cancer cells after applying a high-energy 6 MV X-ray clinical beam. Additionally, the study investigates the dependence of both RIBE in bystander cells and biological responses in target cells on radiation dose by comparing normal and cancer cells in several aspects, including their cell survival and the possible consequences such as DNA damage and cell morphology changes. The study was performed by exposing the

cancerous MCF-7 and normal MCF-10A breast cell lines to varying doses of X-ray ionizing radiation (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 Gy) to observe the biological responses in bystander and targeted cells.

1.5 Organization of the thesis

This thesis is organized into five chapters, each focusing on different aspects of the research. Chapter 1 is the introduction, outlining the study's overview, problem statement, and scope. This chapter also highlights the research objectives and aims.

Chapter 2 reviews the history of radiation, its role in radiation biology and medical applications, and the challenges in radiotherapy, including radioresistance and RIBE. It discusses the mechanisms of radiation injury and its effects on cellular and molecular components, concluding with an exploration of RIBE and the factors influencing its occurrence. Chapter 3 details the methodology employed in this study, including the experimental design, cell culture conditions, radiation exposure protocols, and data analysis techniques used to assess the effects of radiation on both targeted and bystander cells. Chapter 4 presents the results of the experiments, offering a detailed analysis of the data obtained from the experiments conducted on MCF-7 breast cancer cells and MCF-10A normal breast cells. The results are discussed in the context of radiation-induced cytotoxicity, cell viability, and the interplay between targeted and bystander cell responses.

Finally, Chapter 5 presents the conclusion of the study, summarizing the findings and their implications for cancer radiotherapy. It also offers recommendations for future research in radiation biology, with a focus on understanding and modulating radiation-induced bystander effects (RIBE) to enhance therapeutic outcomes while minimizing damage to healthy tissues.

CHAPTER 2

LITERATURE REVIEW

2.1 History of radiation

Henri Becquerel's discovery of radioactivity was a pivotal moment in scientific history, occurring shortly after the discovery of X-rays. Initially, Becquerel hypothesized that phosphorescent materials might emit X-rays when exposed to sunlight, similar to the secondary radiation observed in cathode rays. His experiments with various substances were largely unsuccessful until he tested uranium salts, which emitted rays capable of penetrating black paper, leading to the discovery of radioactivity. This discovery was not only a result of logical experimentation but also serendipitous events that guided Becquerel's research (Jauncey, 1945; Romer, 1964).

This discovery was significant as it demonstrated a novel form of radiation, later designated as radioactivity, intrinsic to certain materials, including uranium (Gregorio & Daniel, 2020; Radvanyi & Villain, 2017). Becquerel's findings established a foundational framework for subsequent research by scientists such as Marie and Pierre Curie, who identified additional radioactive elements, including radium and polonium (Gregorio & Daniel, 2020). The discovery of radioactivity catalyzed a more profound understanding of atomic structure and the nature of elements, thereby influencing subsequent scientific advancements (Allisy, 1996). Although Becquerel's discovery was initially motivated by a hypothesis concerning phosphorescence and X-rays, the unforeseen results from his experiments with uranium salts ultimately led to radioactivity identification (Kipnis, 2000).

The discovery of radioactivity has significantly advanced medicine and industry, offering innovative solutions and enhancing existing practices. The studies of the biological effects of ionizing radiation started after the discovery of X-rays in 1895. The observed effects were skin damage (erythema, dermatitis), hair loss, and lung cancer (Boice et al., 2020; Rios et al., 2024). At the same time, the first attempts to treat cancer with radiation appeared. According to data from these investigations, in 1906, Bergonie and Tribondeau reported that rapidly proliferating cells are more sensitive to the effects of radiation than cells that divide more slowly (Beck-Bornholdt et al., 1997; Vogin & Foray, 2013). As understanding of cell cycle processes increased, it became clear that rapidly dividing cells exhibited rapid radiation damage, explaining their remarkable sensitivity. Muller demonstrated the effect of X-rays on the mutation rates of the Drosophila fruit fly in 1927 (Komel, 2023). This was the first work that outlined a dose-response relationship between radiation administered and an increase in mutation rate.

The medical application of X-rays still plays a crucial role in the diagnosis of various diseases and the treatment of cancer. Artificial radioactive isotopes are also used for diagnosis and sometimes for treatment, as is the natural isotope radium. Radioisotopes are integral to diagnostic imaging, such as PET and SPECT scans, which provide detailed insights into physiological processes. These techniques have improved with advances in molecular biology, allowing for precise imaging and monitoring of diseases like cancer (Banerjee et al., 2022; Schubert et al., 2024). They are also used in targeted therapies, delivering radiation directly to cancer cells and minimizing damage to surrounding healthy tissue. This approach, known as theragnostic, combines therapy and diagnostics to enhance treatment efficacy and monitor therapeutic responses (Banerjee et al., 2022; Schubert et al., 2024).

2.2 Challenges in radiotherapy

The primary goal of radiotherapy is to optimize the delivery of radiation to cancerous cells while minimizing the dose absorbed by adjacent healthy tissues (Baskar et al., 2012). To achieve this, radiotherapy techniques must carefully balance treatment intensity with the body's ability to repair radiation-induced damage in healthy cells. Unlike cancer cells, normal cells typically repair radiation-induced damage more efficiently, enabling them to recover functionality more rapidly. Cancer cells, however, have reduced repair efficacy, leading to differential cell death (Baskar et al., 2012). This differential radiosensitivity between healthy and cancerous cells is fundamental to radiotherapy's success, as it allows clinicians to exploit weaknesses in cancer cells' repair pathways. Significant advancements in radiotherapy delivery over the past two decades have focused on addressing tumor versus normal tissue characteristics, such as through dose fractionation (Schaue & Mcbride, 2015). Although advancements in technology have been made, increasing the radiation dose does not significantly enhance the tumor control probability (TCP) for numerous radioresistant tumors (Dubey et al., 2022; Liu et al., 2018). Consequently, effective tumor control remains limited by the balance between treatment efficacy and minimizing radiation toxicity to normal tissues.

Challenges in radiotherapy are amplified by radioresistance in cancer cells and complex biological factors, like the radiation-induced bystander effects (RIBE) (Heeran et al., 2019). Radioresistance allows cancer cells to repair radiation damage, reducing treatment efficacy and leading to potential relapse (Busato et al., 2022). Meanwhile, RIBE impacts neighboring non-irradiated cells, potentially increasing unintended damage to surrounding healthy tissue (Heeran et al., 2019). Addressing these issues involves a deeper understanding of intercellular signaling pathways and

the tumor microenvironment. Advances in precision treatment and modulation of RIBE could help enhance radiotherapy outcomes while minimizing harm to normal cells.

Despite advances in radiotherapy (RT), challenges in its radiobiological application persist. A primary limitation is the difficulty in differentiating between cancerous and healthy tissues, as both exhibit similar mass energy absorption properties, leading to unintended exposure in non-targeted areas (Baatout, 2023). Additionally, tumors often reside close to critical organs and normal tissues, constraining the precision of radiation delivery (Liu et al., 2018). Intensive investigation of the bystander effect (RIBE) reveals that even non-targeted cells can undergo biological changes due to signals from irradiated cells, further complicating efforts to limit collateral damage (Tang et al., 2023). This phenomenon highlights the need for a refined understanding of intercellular signaling pathways and mechanisms that drive off-target responses in RT to enhance the effectiveness of cancer treatments while protecting healthy tissue (Pouget et al., 2018).

Future research should also explore how different radiation doses influence direct and bystander responses, focusing on dose-effect and dose-response relationships. Understanding these interactions can reveal how low and high doses uniquely impact cancerous and normal cells. By integrating knowledge of dose-specific responses with insights into RIBE mechanisms, researchers can refine therapeutic radiation doses to optimize tumor control, reduce unintended bystander effects, and improve outcomes for healthy tissue protection in radiotherapy.

2.3 The biology of radiation interaction

The interaction of ionizing radiation with biological tissues is a complex process that triggers a cascade of events at the atomic, molecular, and cellular levels. These interactions can result in immediate or delayed biological effects, depending on the radiation type, dose, and the biological context of the exposed tissue. The biological responses to radiation are governed by intricate mechanisms that dictate how cells repair damage, survive, or undergo programmed cell death, ultimately shaping the outcomes of radiation exposure.

The biological effects of radiation are influenced by the dose absorbed and the sensitivity of the affected cells. Ionizing radiation can induce somatic, genetic, deterministic, and stochastic effects, with high doses potentially leading to acute radiation syndrome (ARS) (Mohan & Chopra, 2022). Radiation interacts with DNA either directly or indirectly, with indirect effects often mediated by reactive hydroxyl radicals. These interactions can result in mutations, cell death, or cancer (Mohan & Chopra, 2022). The combination of radiation with other treatments, such as hyperthermia or chemotherapeutic agents, can produce synergistic or antagonistic effects. The effectiveness of these combinations depends on factors like dose rate and intensity, which can either amplify or diminish the biological impact of radiation (Evstratova et al., 2023; Kwon et al., 2023). While much attention is given to the detrimental effects of radiation, it is crucial to acknowledge its beneficial applications, such as cancer treatment through radiotherapy.

Understanding the precise mechanisms of radiation interaction with biological systems is essential for improving therapeutic strategies and developing better

protective measures against radiation exposure. This knowledge provides a foundation for harnessing radiation's potential while mitigating its risks.

2.3.1 Mechanisms of radiation injury and radiobiological principles

Radiobiology, an interdisciplinary field bridging physics and biology, focuses on the interactions and effects of ionizing radiation on cellular and molecular components within living tissues (Reisz et al., 2014). This field encompasses understanding the fundamental mechanisms by which radiation induces biological responses and potential injuries at both cellular and molecular levels (Monaghan et al., 2024). An organism is persistently exposed to ionizing radiation originating from both natural and artificial sources, leading to cellular damage by ionizing atoms and molecules within the cells (Bohm et al., 2010). Radiations with high energy can directly or indirectly compromise the integrity of cellular DNA, affecting cells' division and ultimately impeding proliferation (Baskar et al., 2014; Kumar et al., 2023).

When biological tissues are irradiated, they undergo a series of responses that vary over time and are categorized into physical, chemical, and biological phases as depicted in Figure 2.1. Initially, during the physical phase, high-energy particles from the radiation beam interact with atoms within the tissue. This interaction occurs in an extremely short time, within approximately 10^{-18} seconds, as high-energy-speed radiation traverses' cellular DNA, primarily interacting with orbital electrons. This process can lead to the ionization or excitation of atoms within biological molecules, which is essential in initiating a cascade of chemical reactions (Joiner & van der Kogel, 2009).

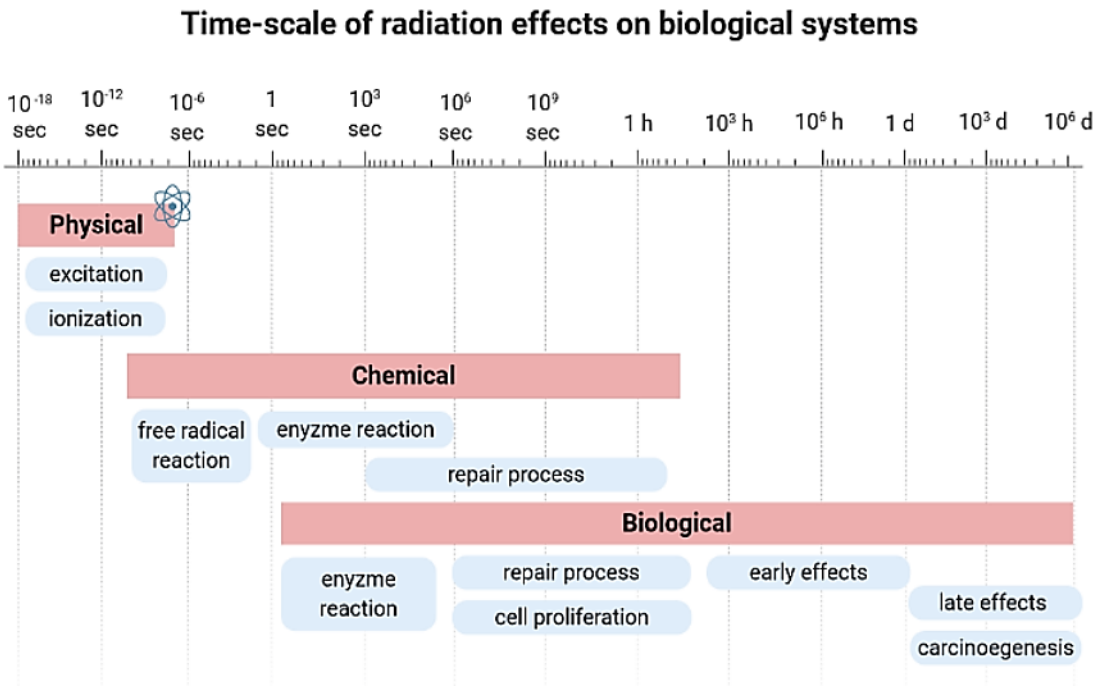

Figure 2.1 Time-scale of the effects of radiation exposure on biological systems.

The chemical phase denotes the stage in which chemical reactions transpire among damaged atoms, molecules, and various cellular components. This phase encompasses the breaking of chemical bonds and the formation of free radicals as a result of excitation and ionization processes. These free radical reactions generally occur within approximately 1 millisecond following irradiation. During this phase, several processes take place, including scavenging reactions that neutralize free radicals and fixation reactions that stabilize molecular chemical alterations (Joiner & van der Kogel, 2009).

The biological period encompasses all subsequent biological responses from ionizing radiation-cell interaction. This effect may result from direct cellular structure interaction, or the effect of indirect interaction mediated by the radiolysis of water. Radiation can cause biological outcomes such as irreparable or improperly repaired DNA damage, leading to death of cells, chromosomal abnormalities, damage in DNA,

arrest in the cell cycle, apoptosis, mutations, and cancer (Jiao et al., 2022). Notably, the effects of ionizing radiation may become evident only years after the initial exposure (Samaila, 2024).

Cellular damage from radiation occurs via two main mechanisms: direct and indirect ionizing radiation actions. These processes predominantly target DNA, leading to cellular injury and potentially resulting in cell death. The primary ionizing radiation impact on tissues is to deplete the populations of cells, which subsequently causes functional impairment due to direct DNA damage and cell destruction (Wilkinson et al., 2023). Figure 2.2 illustrates the indirect and direct mechanisms of damage in DNA induced by radiation.

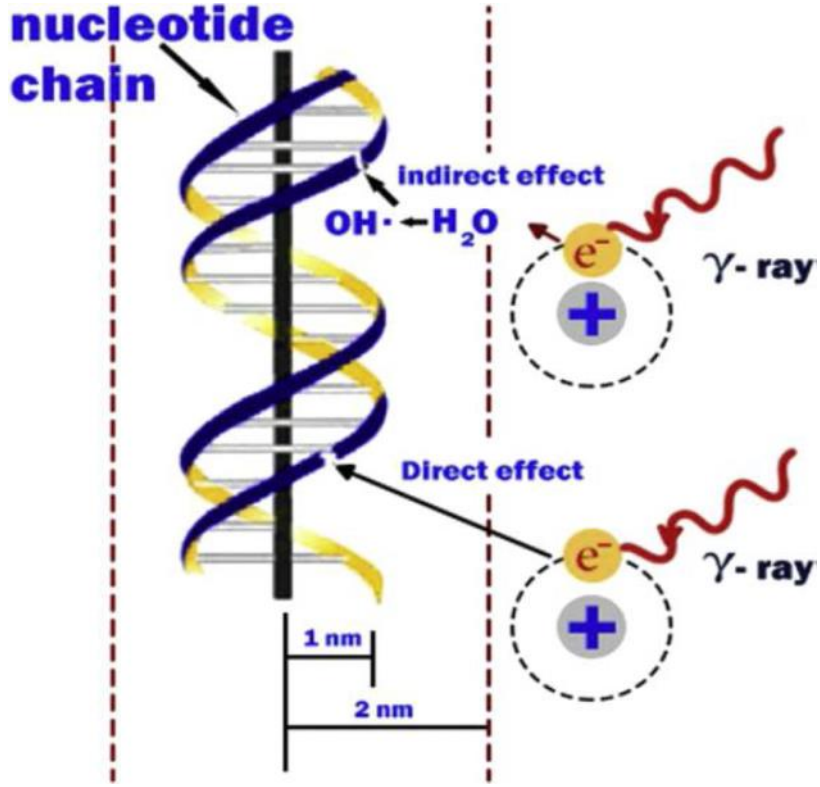

Figure 2.2 The potential mechanisms of radiation-induced injury, including direct and indirect actions, are depicted (adapted from Desouky et al., 2015).

Direct action occurs when ionizing radiation directly strikes and damages critical cellular biomolecules, such as DNA. As shown in Figure 2.3, this interaction can result in DNA single-strand breaks (SSBs), double-strand breaks (DSBs), and structural alterations, particularly cell-lethal (Baudin et al., 2021; Rashid et al., 2022). Damage from direct action is immediate and can lead to chromosomal aberrations and micronuclei formation, both of which are indicators of severe DNA damage (Baudin et al., 2021; Huang & Zhou, 2021). Surviving damaged cells may lead to cancer, aberrations, and mutation (Alhmoud et al., 2020). The ionizing radiation cellular responses are influenced by factors such as the linear energy transfer (LET), radiation energy and type, the administered dose, and the inherent sensitivity of the cells (Maier et al., 2016; Prise et al., 2005; Sutcu et al., 2024; Wilkinson et al., 2023). High-linear energy transfer (LET) radiation, like neutrons and α-particles, primarily induces cellular damage through direct interactions, particularly at higher radiation doses (Desouky et al., 2015).

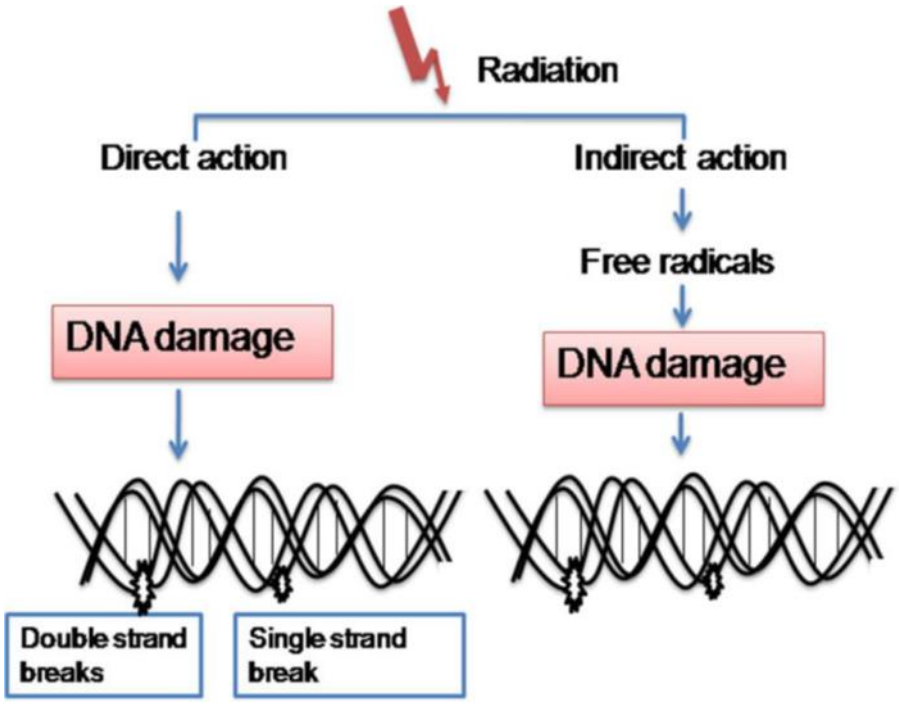

Figure 2.3 Mechanisms of radiation action: direct and indirect pathways (adapted from Baskar et al., 2014).

Indirect action occurs when ionizing radiation interacts with molecules of water within the cell, generating free radicals, such as radical hydroxyl. These highly reactive species can subsequently damage DNA and other cellular components. (Bohm et al., 2010; Rashid et al., 2022). This mechanism is significant because the free radicals generated can perpetuate the radiation effects by interacting with cellular oxygen, further amplifying the damage (Baskar et al., 2014). The indirect action is responsible for a substantial portion of radiation-induced cellular damage due to the abundance of water in cells, making it a critical pathway for radiation injury (Rashid et al., 2022). These operations generate free radical outcomes, including hydroxyl (HO•) and alkoxy (RO2•) radicals. These radicals are capable of diffusing over considerable distances to damage critical cellular targets (Desouky et al., 2015; Bohm et al., 2010). While direct and indirect actions are the primary mechanisms of radiation injury, the severity and type of damage can vary based on factors like the radiation dose, LET, and specific cellular environment. Moreover, the body's capacity to repair radiation-induced damage is a critical factor in influencing the biological outcomes of radiation exposure.

Free radicals are atoms or molecules characterized by unpaired electrons or an uneven electron number in their outer shell (Bohm et al., 2010). These radicals are highly reactive, unstable, and short-lived, readily interacting with other compounds electrons to attain stability. The loss of an electron by a molecule during this interaction transforms it into a free radical, triggering a chain reaction that can result in damage to healthy cells (Phaniendra et al., 2015). Indirect DNA effects of radiation, caused by free radicals, often result in cell dysfunction or death. Preliminary free radicals have a brief lifespan, approximately 1^{-10} seconds (Bohm et al., 2010). In low linear energy transfer (LET) radiation, like gamma and X-rays, indirect mechanisms account for

about 60% of cellular damage due to the high-water content of cells, which makes up roughly 70% of their composition (Russ et al., 2022; Talapko et al., 2024).

Ionizing radiation interacts with biological materials, resulting in the disruption of chemical bonds and the ionization of atoms and molecules. This process affects critical cellular components such as water, DNA, membrane lipids, and proteins (Ibanez et al., 2024; Reisz et al., 2014). This interaction is crucial for understanding the effects of radiation on biological systems and its applications in medical and industrial fields. Ionizing radiation can interact with biological molecules either directly or indirectly, significantly impacting cellular and molecular integrity (Reisz et al., 2014). The process leads to charge separation and ionization, particularly in DNA, where the transfer of excited electrons is influenced by chemical bonding, such as π-π interactions and hydrogen bonds, which affect the extent of ionization (Deng et al., 2023).

Clusters of ionization events along charged particle tracks can result in significant biological effects, including DNA strand breaks and base lesions, which are critical for understanding radiation-induced damage (Kinoshita & Zabarmawi, 2020).

Various DNA lesions can occur to the DNA of cells irradiated with X-rays, including single-strand breaks (SSBs), double-strand breaks (DSBs), crosslinks of protein-DNA, damage of base, and crosslinks of protein-protein (Bohm et al., 2010; Wilkinson et al., 2023). These lesions disrupt critical cellular functions, interfere with replication and transcription, and, if left unrepaired or misrepaired, can cause mutations, instability of the genome, or death of cells. SSBs involve damage to the phosphodiester backbone of a single DNA strand. Although cells typically repair SSBs, errors in the repair process may lead to mutations or base mismatches (Rosenblatt et

al., 2018; Wilkinson et al., 2023). In contrast, DSBs occur when the breaks happen in both strands of the DNA, either in direct opposition to each other or only a few base pairs apart. While double-strand breaks occur approximately 0.04 times less frequent than SSBs, their occurrence increases linearly with radiation dose (Bohm et al., 2010). It is estimated that exposure to 1 Gy of radiation can induce between 20 and 40 double-strand breaks per cell, and double-strand breaks unrepaired are strongly associated with the lethality of cellular (Golden et al., 2012).

Radiation exposure can elicit a range of cellular consequences, including mitotic or division delays, apoptosis, failure in reproduction, instability in genomics, mutations, transformation of cells, RIBE, and responses-adaptive (Jiao et al., 2022a; Mavragani et al., 2017). Irradiated cells may experience disruptions in normal cell division, leading to mitotic delays. Apoptosis may occur if a cell undergoes programmed death before completing division, often resulting in fragmentation into smaller apoptotic bodies and by neighboring cells-absorbed (Jiao et al., 2022a). Failure of reproduction arises at the time cells cannot survive the first or subsequent mitotic events (Levine & Holland, 2018). Additionally, radiation-induced genomic instability can contribute to reproductive failure and may persist in surviving cells, potentially leading to mutations that affect progeny. Mutations in irradiated cells can result in phenotypic transformations, potentially driving carcinogenesis (Huang et al., 2003; Podgorsak, 2005; Zhang et al., 2024).

Furthermore, RIBE manifests when irradiated cells transmit signals to adjacent or neighboring cells, inducing biological changes in non-irradiated cells. Adaptive responses may also occur, wherein irradiated cells may develop raised resistance to following exposures of radiation, illustrating the complexity of cellular responses to ionizing radiation (Buonanno et al., 2023; Podgorsak, 2005).

Cell irradiation can result in various cellular outcomes, each driven by distinct mechanisms and implications. The nature and severity of these effects are influenced by factors such as radiation dose, type, and cellular context (Sagkrioti et al., 2022).

Ionizing radiation can induce genomic instability, characterized by nonclonal genomic damage in progeny cells. This instability is often associated with centrosome deregulation, leading to conditions such as tetraploidy and aneuploidy (C. A.; Maxwell et al., 2023). High-dose radiation can cause severe DNA damage, including both DNA strand breaks (single and double), which, if not repaired properly, may result in mutations and carcinogenesis (Jia et al., 2021).

Radiation-induced cell death occurs through various mechanisms, including apoptosis, necrosis, and autophagy-dependent cell death, as shown in Figure 2.4. The specific pathway activated depends on the cellular environment and the dose of radiation (Jia et al., 2021). Apoptosis, a common response to persistent DNA damage, serves as a protective mechanism to eliminate damaged cells (Carlos-Reyes et al., 2021).

Cells may exhibit adaptive responses to low-dose radiation, enhancing their ability to repair subsequent damage. This dose-dependent response can significantly influence overall cellular outcomes (Jia et al., 2021). Bystander effects, where non-irradiated cells exhibit radiation-like damage due to signals received from irradiated cells, contribute to genomic instability and other cellular changes (Zhang et al., 2022).

Radiation can also lead to reproductive failure by disrupting cell division processes, often through DNA damage-induced cell cycle arrest, resulting in mitotic delays (Carlos-Reyes et al., 2021). While radiation can induce significant cellular damage, some cells develop resistance mechanisms, such as enhanced DNA repair

pathways, which may lead to radioresistance and affect the efficacy of radiotherapy in cancer treatment. Understanding these mechanisms is essential for advancing therapy strategies and optimizing medicinal outcomes (Carlos-Reyes et al., 2021).

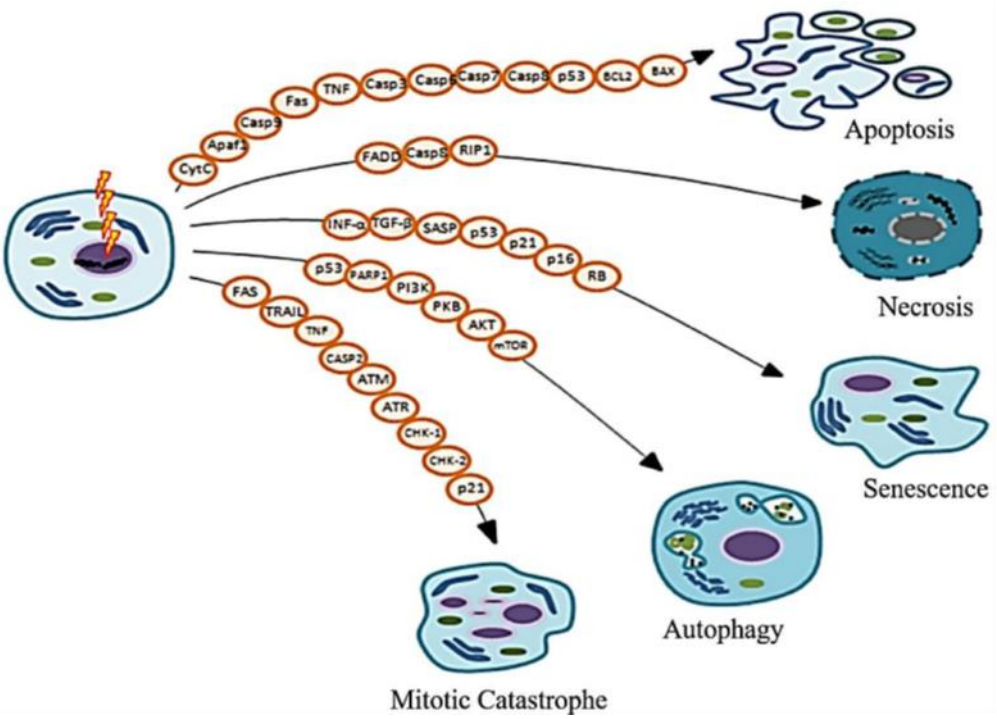

Figure 2.4 The depiction of various mechanisms underlying radiation-induced cell death (adapted from Minafra & Bravatà, 2014).

Radiation-induced cellular damage can be largely mitigated through various enzymatic DNA repair mechanisms. Repair processes aim to restore the functionality of macromolecules, although rejoining DNA strand breaks does not always ensure the restoration of gene function. In some cases, rejoining can introduce genetic defects or mutations into the cell (Joiner & van der Kogel, 2009; Yousaf & Nasir, 2024). Recovery of cells or tissue denotes the enhancement in the survival of cells or the mitigation of radiation-induced damage, provided adequate time is given for recovery mechanisms to take effect (Kim et al., 2014).

DNA repair in cells encompasses various enzymatic mechanisms, each specifically adapted to rectify distinct types of lesions. DSBs are predominantly patched through two key mechanisms: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ operates by ligating blunt-ended DNA fragments generated by the severance of phosphodiester bonds. This pathway is active throughout the cell cycle but is particularly pronounced during the G1 and S phases. Nevertheless, NHEJ is susceptible to errors owing to its lack of dependence on sequence homology (Hall & Giaccia, 2019; Huang & Zhou, 2020; Li, 2017; Li et al., 2024).

In contrast, homologous recombination (HR) employs sequence homology from an intact template to precisely restore the damaged region. This pathway is confined to the late cell cycle phases (S and G2), during which a chromatid of the sister is accessible. Furthermore, other repair mechanisms—including base excision repair (BER), mismatch repair (MR), and nucleotide excision repair (NER)—target distinct types of damage, like oxidation of base, alkylation, and intercalation of strands (Hall & Giaccia, 2019; Bohm et al., 2010; Li et al., 2024; Wendy & Pederson, 2017).

2.3.2 Radiation-induced non-targeted effects

Historically, the ionizing radiation effects were primarily attributed to the direct cellular ionization structures, specifically DNA, or indirect damage caused by ROS generated during the radiolysis of water (Ibanez et al., 2024; Reisz et al., 2014). These biological effects were believed to be localized within the tissue and the targeted area cells. Nonetheless, this perspective is being reevaluated in light of evidence concerning radiation-induced bystander effects (RIBE), also known as non-targeted

effects (Heeran et al., 2019; Lyng & Azzam, 2024). This phenomenon suggests that ionizing radiation can exert influences on neighboring unirradiated tissue or cells.

As shown in Figure 2.5, the ionizing radiation impact on treated cells and its subsequent effects on unirradiated cells, referred to as the bystander effect, has been the focus of investigation for over a century. The bystander effect encompasses the radiation-induced responses observed in non-irradiated cells that are in proximity to irradiated cells.

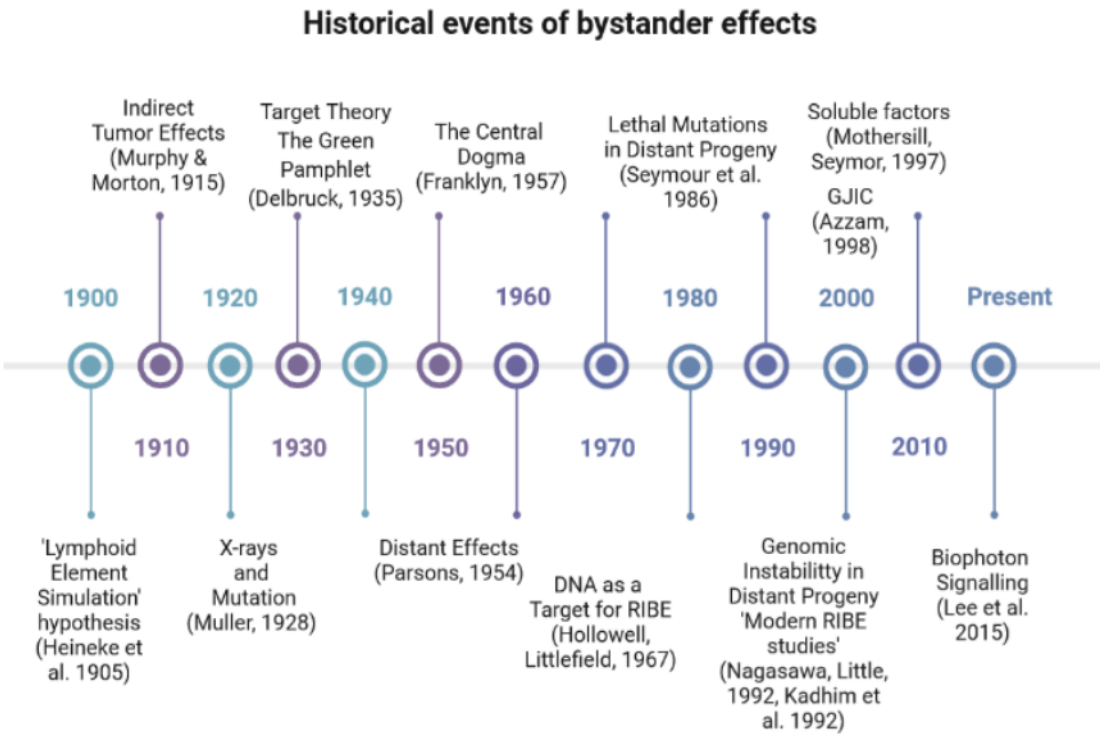

Figure 2.5 A Century-Long Timeline of Research on Bystander Effects (adapted from Mothersill et al., 2018).

In addition to bystander effects, radiation-induced signaling can be classified into two other categories: effects of abscopal and cohort (Wang, 2018; Tang et al., 2023). These classifications are illustrated in Figure 2.6. The abscopal effect refers to a phenomenon where radiation triggers cell responses far from the irradiated field (Janopaul-Naylor et al., 2021; Lyng & Azzam, 2024; Pouget et al., 2018; Reynders et

al., 2016). This effect occurs when irradiated tissues transmit signals to non-targeted areas (Daguenet et al., 2020; Mothersill et al., 2018; Wang, 2018).

While the effect of abscopal is associated with clinical changes resulting from radiation exposure, the RIBE specifically involves radiobiological consequences in non-targeted areas near irradiated tissues (Lyng & Azzam, 2024; Moloudi et al., 2023). The effect of abscopal is commonly observed in patients with metastatic cancers undergoing radiotherapy, where localized irradiation leads to damage of chromosomes and cellular changes in non-targeted areas (Moloudi et al., 2023; Wang, 2018; Reynders et al., 2016).

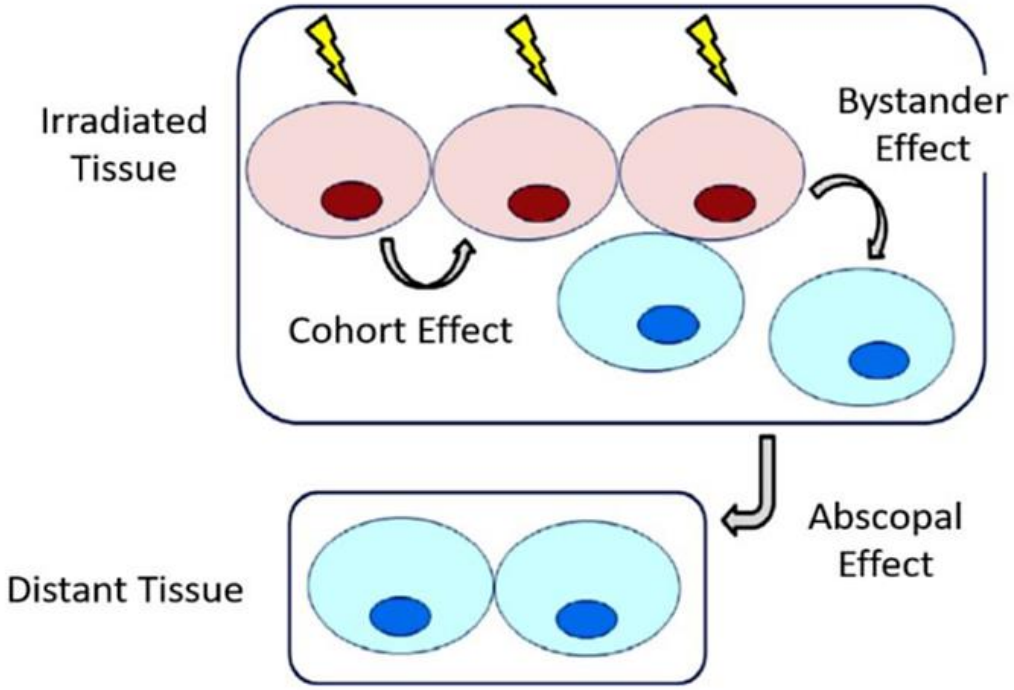

Figure 2.6 A schematic depiction of radiation-induced signaling effects in both targeted and bystander cells, with irradiated cells represented in red and non-irradiated cells depicted in blue (adapted from Butterworth et al., 2013).