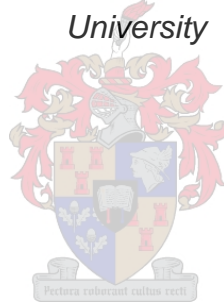


**EVALUATION OF THE EFFECT OF RADIOFREQUENCY ELECTROMAGNETIC
WAVES ON RADIOSENSITIVITY**

Angela Chinhengo

*Thesis presented in partial fulfilment of the requirements for
the degree of Doctor of Philosophy in Radiobiology in the
Faculty of Medicine and Health Sciences at Stellenbosch
University*



Supervisor: Prof JM Akudugu

Co-supervisor: Dr AM Serafin

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2019

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Abstract

Cancer is a major cause of human death worldwide, and one of the very real challenges is how to control treatment resistance. An additional challenge is the co-morbidity of cancer, with certain infections complicating its management. Radiotherapy (RT) is considered the first line of treatment for most superficial cancers, as these malignancies tend to respond well to radiation. The use of hypofractionated treatment may be beneficial for certain tumours, but hypofractionation may result in severe side-effects from normal tissue toxicity from which the patient may not recover. To circumvent this, radiation modifying agents that potentiate the tumour inactivating effects of ionising radiation and thereby lead to a reduction in radiation dose and prevent normal tissue toxicity, can be utilised. Magnetic fields have long been suggested as potential enhancers of radiation effects. Studies on the combined biological effects of radiofrequency fields (RFF) and ionising radiation are virtually non-existent. The use of RFF adjuvant to radiotherapy may be beneficial, as they have been shown to exhibit *in vitro* radiosensitising and radioprotective effects in malignant and normal cells, respectively, with the possibility of a significant dose reduction. There is, however, a need to understand the mechanisms by which these RFF influence radiosensitivity so that they can be employed efficiently as radiotherapy modulators. The main goal of radiotherapy is to kill tumour cells and spare normal tissue, and a good modifying agent would be one that sensitises the tumour whilst protecting normal tissue.

This study assessed the effect of radiofrequency fields (RFF), modulated at 100, 1000, 2000 and 4000 Hz, on the radiosensitivity of four cell lines: a p53 mutant melanoma

cell line, MeWo; a p53 wild-type melanoma cell line, Be11; a p53 mutant prostate cancer cell line, DU145; and a p53 wild-type normal lung fibroblast cell line, L132. The radiomodulatory effect of radiofrequency fields was evaluated using the colony assay. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), superoxide dismutase (SOD) and micronucleus assays were used to assess the possible mechanisms by which radiofrequency fields influence the radiosensitivity of cells.

The data demonstrate that radiofrequency fields are more efficient in modulating large fractional doses of X-rays and could find application in hypofractionated radiotherapy as adjuvants, especially for tumours with low alpha/beta ratios. This can have a positive impact on the management of patients with superficial tumours that may be resistant to low fractional doses of radiation. Radiofrequency fields modulate cellular radiosensitivity in a frequency- and cell type-dependent manner and their effects appear to be linked to p53 status. Cellular responses such as metabolism, DNA damage processing (based on micronuclei formation), and abnormal proliferation (based on binucleation) seem to be underlying factors mediating the radiomodulatory effects of radiofrequency fields.

Mechanisms by which radiofrequency fields can possibly modulate radiosensitivity are: amplification of radiation-induced genotoxicity, cell cycle arrest, and disturbance of other cellular biochemical processes that lead to alteration of homeostasis. Alternative ways by which RFF affect radiosensitivity are: interfering with the synthesis and function of charged proteins in the cell leading to programmed cell death or premature cell ageing, perturbation of intracellular calcium ions which can trigger

apoptotic or necrotic cell death, and/or modulating the expression of Bcl-2 family proteins.

Given this complexity, a potential use of radiofrequency fields as a non-invasive therapeutic modality would require standardisation to establish reproducibility. A more detailed understanding of how radiofrequency fields interact with ionising radiation would also prove beneficial in the broader field of radiation protection.

Opsomming

Kanker is wêreldwyd 'n belangrike oorsaak van die dood in mense, en een van die werklike uitdagings is hoe om weerstand teen behandeling te voorkom. 'n Addisionele uitdaging is dat die komorbiditeit van kanker en infeksies die behandeling bemoeilik. Bestralingsterapie word by voorkeur as die eerste keuse van behandeling vir die meeste van die oppervlakkige kankers beskou, want hierdie kwaadaardige siektes is kwesbaar vir bestraling. Die gebruik van hipofraksioneerde behandeling mag voordelig vir die behandeling van sekere tumore wees, maar hipofraksionering mag moontlik bykomstige nadelige gevolge in die vorm van onomkeerbare lewensbedreigende toksisiteit inhou. Om dit te voorkom, kan stralingsmodifiërende strategieë gebruik word, met die vermoë om die tumorinaktivering te potensieer, bestralingsdosis te verlaag en weefseltoksisiteit van normale weefsel te verminder. Magnetiese velde word lankal as 'n potensiële versterker van stralingseffekte beskou. Studies oor die gekombineerde biologiese uitwerking van radiofrekwensievelde (RFV) en gëioniseerde bestraling is selde gerapporteer. Die benutting van radiofrekwensievelde saam met bestraling mag voordelig wees, want dit is bewys dat hierdie kombinasie terapie *in vitro* daarin slaag om maligne en normale selle teen bestralingskade te beskerm, omdat die bestralingsdosis aansienlik verlaag kan word. Dit is egter nodig dat die meganismes, waardeur radiofrekwensievelde die bestralings sensitiviteit beïnvloed, opgeklaar word sodat dit voordelig as modulators van radioterapie aangewend kan word. Die hoofdoel van radioterapie is om tumorselle dood te maak en om normale selle te beskerm. 'n Nuttige modulator is dié wat normale selle beskerm, terwyl dit tumorselle kwesbaar maak.

Hierdie studie het die effek van radiofrekwensievelde (RFV) gemoduleerd met 100, 1000, 2000, en 4000 Hz, op die verhouding van die radiosensitiwiteit van vier sellyne evalueer: 'n p53-mutant melanoomsellyn, MeWo; 'n p53-wildetipe melanoomsellyn, Be11; 'n p53-mutant prostaatkankersellyn, DU145; en 'n p53-wildetipe normale longsellyn, L132. Die stralingsregulerende effek van radiofrekwensievelde is bereken deur die gebruik van kolonie-essaiëring. Die MTT, SOD en mikrokerntoetse is gebruik om die moontlike meganismes waardeur radiofrekwensievelde die radiosensitiwiteit van selle beïnvloed, te bepaal.

Die data bewys dat radiofrekwensievelde meer doeltreffend is wanneer dit toegepas word op modulering van groot gefraksioneerde dosisse X-strale en mag dien as bykomende hipofraksioneerde stralingsterapie, veral in tumore met lae alfa/beta-verhoudings. Hierdie addisionele voordeel kan verder benut word by die behandeling van pasiënte met oppervlakkige tumore wat weerstandig is teen lae gefraksioneerde stralingsdosisse. Radiofrekwensievelde moduleer die radiosensitiwiteit van selle op 'n frekwensie- en seltipe-afhanklike wyse en die uitwerking blyk gekoppel te wees aan p53-status. Sellulêre reaksies soos metabolisme, DNS-skadeprosessering (gebaseer op mikrokernvorming), en abnormale proliferasie (gebaseer op dubbelkernvorming) skyn onderliggende faktore te wees wat die radiomodulerende effekte van radiofrekwensievelde medieer.

Meganismes waardeur radiofrekwensievelde moontlik die radiosensitiwiteit kan reguleer, is: versterking van bestralingsgeïnduseerde geentoksisiteit, selsiklusarres, en versteuring van ander sellulêre biochemiese prosesse wat lei tot versteuring van homeostase. Alternatiewe meganismes waardeur RFV radiosensitiwiteit beïnvloed, is:

versteuring van die sintese en funksionering van intrasellulêre polêre proteïene wat lei tot geprogrammeerde seldood of premature veroudering van selle, ontwrigting van intrasellulêre kalsiumioonekwilibrium wat apoptose of nekrotiese seldood kan sneller en/of die uitdrukking van Bcl-2- familieproteïene kan moduleer.

Teen die agtergrond van hierdie ingewikkelde oorwegings, verg die potensiële benutting van radiofrekwensievelde as 'n nie-indringende terapeutiese modulator standaardisasie om herhaalbaarheid vas te stel. 'n Meer gedetailleerde begrip van hoe presies die interaksie van radiofrekwensievelde met geïoniseerde bestraling verklaar kan word, mag ook van toepassing wees op die gebied van beskerming teen bestraling op velerlei ander gebiede.

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Opinions expressed and the conclusions arrived at in this dissertation are those of the author, and are not necessarily to be attributed to the Faculty of Medicine and Health Sciences (Stellenbosch University), the NRF or the Harry Crossley Foundation.

Dedications

I dedicate this study to my children, whose love kept me going and whose presence gave me strength.

CONTENT	PAGE
<i>Declaration</i>	ii
<i>Abstract</i>	iii
<i>Opsomming</i>	vi
<i>Acknowledgements</i>	ix
<i>Dedication</i>	x
<i>List of Tables</i>	xiv
<i>List of Figures</i>	xvi
<i>List of Abbreviations</i>	xx
CHAPTER 1	1
1.1. Introduction and Rationale	2
1.2. Literature Review	7
1.2.1. Radiation Therapy Overview	7
1.2.2. Targeted Therapy	10
1.2.3. Combination Therapy	11
1.2.4. The Co-morbidity of Cancer and Other Infections, and the Possible Influence of EMF on the Management of Such Cancers	13
1.2.5. Effects of Electromagnetic Fields	15
1.2.5.1. <i>Electric Fields</i>	15
1.2.5.2. <i>Magnetic Fields</i>	18
1.2.5.3. <i>Radiofrequency Fields</i>	20
1.3. Problem Statement	24
1.4. Research Question	25
1.5. Hypothesis	26
1.6. Aims and Objectives	27

1.7. Delineations	28
1.8. Limitations	29
CHAPTER 2	30
2. Materials and Methods	31
2.1. Study Location	31
2.2. Cell Lines and Culture Maintenance	31
2.3. Preparation of Cell Cultures for Experiments	33
2.4. Irradiation of Cell Cultures	33
2.5. Radiofrequency Generation and Exposure	34
2.6. Clonogenic Cell Survival Assay	38
2.7. Effect of Radiofrequency Fields on Plating Efficiency	40
2.8. Radiomodulatory Effects of Radiofrequencies	40
2.9. Assessment of Metabolic Activity	41
2.10. Measurement of Cytosolic Superoxide Dismutase (SOD) Activity	43
2.11. Assessment of DNA Damage Response	44
2.12. Data Analysis	46
CHAPTER 3	47
3. Results	48
3.1. Effect of Radiofrequency Field Exposure on Plating Efficiency	48
3.2. Radiosensitivity and Radiomodulatory Effect of Radiofrequency Fields	49
3.3. Summary of Relative Cellular Radiosensitivity	60
3.4. Effect of Radiofrequency Fields Modulated at Higher Frequencies on Radiosensitivity	62
3.5. Effect of Radiofrequency Fields on Radiation-Induced Metabolic Changes	66
3.6. Relationship between Radiosensitivity and Metabolic Activity	74

3.7. Effect of a Radiofrequency Field on Radiation-Induced Changes in Superoxide Dismutase (SOD) Activity	77
3.8. Relationship between Radiosensitivity and SOD Activity	83
3.9. Effect of Radiofrequency Fields on Radiation-Induced Micronucleus Frequency	84
3.10. Relationship between Radiosensitivity and Micronucleus Yield	96
3.11. Relationship between Radiosensitivity and Binucleation	98
CHAPTER 4	100
4. Discussion	101
4.1. Intrinsic Radiosensitivity and Radiomodulatory Effect of Radiofrequency Fields	102
4.2. Effect of Radiofrequency Fields on Radiation-Induced Changes in Metabolic Activity and its Relation to Overall Cell Survival	105
4.3. Effect of Radiofrequency Fields on Radiation-Induced Changes in Superoxide Dismutase (SOD) Activity and its Relation to Overall Cell Survival	107
4.4. Effect of Radiofrequency Fields on Radiation-Induced Micronucleus Frequency, and the Possible Impact on Cell Survival	108
4.5. Effect of Radiofrequency Fields on Radiation-Induced Changes in Binucleation	110
CHAPTER 5	113
5. Conclusion	114
Possible Future Avenues	116
References	118
Appendices	149

LIST OF TABLES

Table 2.1: Estimated peak electric field (E), magnetic flux density (B), and current density (J) induced at a distance (d) from the axis of a 29-cm plasma ray tube	37
Table 2.2: Summary of cell numbers seeded at each radiation dose of X-rays delivered acutely	38
Table 3.1: Summary of plating efficiency modifying factors for DU145, MeWo, Be11, and L132 cell lines following exposure to 100, 1000, 2000, and 4000-Hz modulated radiofrequency fields. Data are presented as the mean \pm SEM from 3 independent experiments	48
Table 3.2: Summary of radiobiological parameters for the DU145 cell line. SF_2 and SF_6 denote the surviving fraction at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. \bar{D} denotes the mean inactivation dose (area under the cell survival curve). Data are presented as the mean \pm SEM from 3 independent experiments	50
Table 3.3: Modifying factors (MF), relative to X-ray treatment alone, derived from the SF_2 , SF_6 , and \bar{D} values presented in Table 3.2 for the DU145 cell line according to Equation (2.4). Errors were calculated using error propagation formulae for ratios	51
Table 3.4: Summary of radiobiological parameters for the MeWo cell line. SF_2 and SF_6 denote the surviving fraction at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. \bar{D} denotes the mean inactivation dose (area under the cell survival curve). Data are presented as the mean \pm SEM from 3 independent experiments	53
Table 3.5: Modifying factors (MF), relative to X-ray treatment alone, derived from the SF_2 , SF_6 , and \bar{D} values presented in Table 3.4 for the MeWo cell line according to Equation (2.4). Errors were calculated using error propagation formulae for ratios	54
Table 3.6: Summary of radiobiological parameters for the Be11 cell line. SF_2 and SF_6 denote the surviving fraction at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. \bar{D} denotes the mean inactivation dose (area under the cell survival curve). Data are presented as the mean \pm SEM from 3 independent experiments	56
Table 3.7: Modifying factors (MF), relative to X-ray treatment alone, derived from the SF_2 , SF_6 , and \bar{D} values presented in Table 3.6 for the Be11 cell line according to Equation (2.4). Errors were calculated using error propagation formulae for ratios	57
Table 3.8: Summary of radiobiological parameters for the L132 cell line. SF_2 and SF_6 denote the surviving fraction at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. \bar{D} denotes the mean inactivation dose (area under the cell survival curve). Data are presented as the mean \pm SEM from 3 independent experiments	59

- Table 3.9:** Modifying factors (MF), relative to X-ray treatment alone, derived from the SF_2 , SF_6 , and \bar{D} values presented in Table 3.8 for the L132 cell line according to Equation (2.4). Errors were calculated using error propagation formulae for ratios 60
- Table 3.10:** Summary of relative radiosensitivity of DU145, L132, Be11, and MeWo cell lines based on SF_2 , SF_6 , and \bar{D} 61
- Table 3.11:** Modifying factors (MF), relative to X-ray treatment alone, derived from the relative metabolic activities presented in Figure 3.9 for the DU145 cell line, as described in Section 2.9. Errors were calculated using error propagation formulae for ratios 68
- Table 3.12:** Modifying factors (MF), relative to X-ray treatment alone, derived from the relative metabolic activities presented in Figure 3.10 for the MeWo cell line, as described in Section 2.9. Errors were calculated using error propagation formulae for ratios 70
- Table 3.13:** Modifying factors (MF), relative to X-ray treatment alone, derived from the relative metabolic activities presented in Figure 3.11 for the Be11 cell line, as described in Section 2.9. Errors were calculated using error propagation formulae for ratios 72
- Table 3.14:** Modifying factors (MF), relative to X-ray treatment alone, derived from the relative metabolic activities presented in Figure 3.12 for the L132 cell line, as described in Section 2.9. Errors were calculated using error propagation formulae for ratios 74
- Table 3.15:** Modifying factors (MF), relative to X-ray treatment alone, derived from the superoxide dismutase (SOD) activities presented in Figures 3.16-3.19 for the DU145, MeWo, Be11, and L132 cell lines, as described in Section 2.10. Errors were calculated using error propagation formulae for ratios 82
- Table 3.16:** Modifying factors (MF), relative to X-ray treatment alone, derived from the micronucleus frequency presented in Figures 3.21, 3.23, 3.25, and 3.7 for the DU145, MeWo, Be11, and L132 cell lines, respectively, as described in Section 2.11. Errors were calculated using error propagation formulae for ratios 95
- Table 3.17:** Modifying factors (MF), relative to X-ray treatment alone, derived from the binucleation indices presented in Figures 3.22, 3.24, 3.26, and 3.8 for the DU145, MeWo, Be11, and L132 cell lines, respectively, as described in Section 2.11. Errors were calculated using error propagation formulae for ratios 98

LIST OF FIGURES

- Figure 1.1:** Radiotherapy-induced skin burns on a patient treated for carcinoma of the base of the tongue (from Lee et al., 2002) 4
- Figure 1.2:** Example of radiotherapy-induced late effects and ulceration (from Jacobson et al., 2017) 4
- Figure 1.3:** Radiation interacts with cellular DNA directly or indirectly (adapted from Basker et al., 2012) 7
- Figure 1.4:** Radiation damage triggers a signal that is relayed from ATM to p53, leading to cell death (adapted from Cohen-Jonathan et al., 1999) 8
- Figure 1.5:** HHV-8 associated Kaposi's Sarcoma (National Cancer Institute visuals online, 2001; <https://visualsonline.cancer.gov/details.cfm?imageid=2168>) 13
- Figure 1.6:** HPV-mediated genital warts associated with cervical cancer (Feigwarzen Bilder; <https://www.warzen-hilfe.com/feigwarzen-welcher-arzt/>) 14
- Figure 1.7:** The Fenton reaction: conversion of hydrogen peroxide (H_2O_2) into a highly reactive hydroxyl radical (OH^*) in the presence of iron (Phillips et al., 2008) 23
- Figure 2.1:** Photograph of the Precision MultiRad 160 X-ray irradiator (door opened) showing cell culture flasks on the turntable 34
- Figure 2.2:** (A) Photograph of the electromagnetic field (EMF) exposure system, with the PERL M⁺ inverted on an appropriately cut styrofoam box. (B) A 2-dimensional schematic diagram showing the top and bottom cell culture planes of the 2×2×4 flask matrix. In the set-up, the plasma ray tube is centred horizontally above the cell culture flasks, such that the induced magnetic field (B) is parallel to the base of a flask and the induced electric field (E) in the culture medium is parallel to the width of the flask 35
- Figure 3.1:** Clonogenic survival curves for the DU145 cell line after X-ray irradiation alone (black) and in combination with 100-Hz (A) and 1000-Hz (B) modulated radiofrequency fields (RFF). RFF exposure was performed 2 h prior to (blue) or after (red) X-ray treatment. The survival curves were obtained by fitting data from three independent experiments to the linear-quadratic model (Equation 2.2) 49
- Figure 3.2:** Clonogenic survival curves for the MeWo cell line after X-ray irradiation alone (black) and in combination with 100-Hz (A) and 1000-Hz (B) modulated radiofrequency fields (RFF). RFF exposure was performed 2 h prior to (blue) or after (red) X-ray treatment. The survival curves were obtained by fitting data from three independent experiments to the linear-quadratic model (Equation 2.2) 52
- Figure 3.3:** Clonogenic survival curves for the Be11 cell line after X-ray irradiation alone (black) and in combination with 100-Hz (A) and 1000-Hz (B) modulated radiofrequency fields (RFF). RFF exposure was performed 2 h prior to (blue) or after (red) X-ray treatment. The survival

curves were obtained by fitting data from three independent experiments to the linear-quadratic model (Equation 2.2) 55

- Figure 3.4:** Clonogenic survival curves for the L132 cell line after X-ray irradiation alone (black) and in combination with 100-Hz (A) and 1000-Hz (B) modulated radiofrequency fields (RFF). RFF exposure was performed 2 h prior to (blue) or after (red) X-ray treatment. The survival curves were obtained by fitting data from three independent experiments to the linear-quadratic model (Equation 2.2) 58
- Figure 3.5:** Cell survival data of the DU145 cell line for various radiofrequency field treatments at (A) 2 Gy and (B) 6 Gy 62
- Figure 3.6:** Cell survival data of the MeWo cell line for various radiofrequency field treatments at (A) 2 Gy and (B) 6 Gy 63
- Figure 3.7:** Cell survival data of the Be11 cell line for various radiofrequency field treatments at (A) 2 Gy and (B) 6 Gy 64
- Figure 3.8:** Cell survival data of the L132 cell line for various radiofrequency field treatments at (A) 2 Gy and (B) 6 Gy 65
- Figure 3.9:** Relative metabolic activities for the prostate cancer cell line, DU145: (A) 30 min and (B) 18 h after treatment to 6 Gy of X-rays alone or in combination with 100-Hz and 1000-Hz modulated radiofrequency fields 67
- Figure 3.10:** Relative metabolic activities for the melanoma cell line, MeWo: (A) 30 min and (B) 18 h after treatment to 6 Gy of X-rays alone or in combination with 100-Hz and 1000-Hz modulated radiofrequency fields 69
- Figure 3.11:** Relative metabolic activities for the melanoma cell line, Be11: (A) 30 min and (B) 18 h after treatment to 6 Gy of X-rays alone or in combination with 100-Hz and 1000-Hz modulated radiofrequency fields 71
- Figure 3.12:** Relative metabolic activities for human lung fibroblasts, L132: (A) 30 min and (B) 18 h after treatment to 6 Gy of X-rays alone or in combination with 100-Hz and 1000-Hz modulated radiofrequency fields 73
- Figure 3.13:** Plot of modifying factors from metabolic activity (measured 30 min after treatment) as a function of modifying factors from clonogenic cell survival for 4 cell lines: (A) combined treatment with 100-Hz modulated RFF and (B) combined treatment with 1000-Hz modulated RFF. Dashed lines represent the 95% confidence interval 75
- Figure 3.14:** Plot of modifying factors from metabolic activity (measured 18 h after treatment) as a function of modifying factors from clonogenic cell survival for 4 cell lines: (A) combined treatment with 100-Hz modulated RFF and (B) combined treatment with 1000-Hz modulated RFF. Dashed lines represent the 95% confidence interval 76
- Figure 3.15:** Standard curve used to determine the concentration of cytosolic superoxide dismutase (SOD) from absorbance measurements 77
- Figure 3.16:** Superoxide dismutase concentration in DU145 cells following RFF exposure and X-ray irradiation singly or in combination. X-ray treatment was compared with negative control (medium; red horizontal line) and X-ray+RFF treatment was compared with positive control

(1000 Hz; blue horizontal line), to generate relative SOD activities which were used to derive modifying factors, as described in Section 2.10 78

Figure 3.17:Superoxide dismutase concentration in MeWo cells following RFF exposure and X-ray irradiation singly or in combination. X-ray treatment was compared with negative control (medium; red horizontal line) and X-ray+RFF treatment was compared with positive control (1000 Hz; blue horizontal line), to generate relative SOD activities which were used to derive modifying factors, as described in Section 2.10 79

Figure 3.18:Superoxide dismutase concentration in Be11 cells following RFF exposure and X-ray irradiation singly or in combination. X-ray treatment was compared with negative control (medium; red horizontal line) and X-ray+RFF treatment was compared with positive control (1000 Hz; blue horizontal line), to generate relative SOD activities which were used to derive modifying factors, as described in Section 2.10 80

Figure 3.19:Superoxide dismutase concentration in L132 cells following RFF exposure and X-ray irradiation singly or in combination. X-ray treatment was compared with negative control (medium; red horizontal line) and X-ray+RFF treatment was compared with positive control (1000 Hz; blue horizontal line), to generate relative SOD activities which were used to derive modifying factors, as described in Section 2.10 81

Figure 3.20:Plot of modifying factors from superoxide dismutase activity (measured 30 min after treatment) as a function of modifying factors from clonogenic cell survival for 4 cell lines, following combined treatment with a 1000-Hz modulated RFF. Dashed lines represent the 95% confidence interval 83

Figure 3.21:Micronucleus yield in the prostate cancer cell line, DU145, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field 85

Figure 3.22:Binucleation index in the prostate cancer cell line, DU145, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field 86

Figure 3.23:Micronucleus yield in the melanoma cell line, MeWo, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field 87

Figure 3.24:Binucleation index in the melanoma cell line, MeWo, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field 88

Figure 3.25:Micronucleus yield in the melanoma cell line, Be11, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field 90

Figure 3.26:Binucleation index in the melanoma cell line, Be11, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field 91

Figure 3.27: Micronucleus yield in the normal lung fibroblasts, L132, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field
93

Figure 3.28: Binucleation index in the normal lung fibroblasts, L132, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field
94

Figure 3.29: Plot of modifying factors from micronucleus frequency (MNF) at 2 Gy as a function of modifying factors from surviving fraction at 2 Gy for 4 cell lines: (A) combined treatment with 100-Hz modulated RFF and (B) combined treatment with 1000-Hz modulated RFF. Dashed lines represent the 95% confidence interval
97

Figure 3.30: Plot of modifying factors from binucleation index (BNI) at 2 Gy of X-rays as a function of modifying factors from surviving fraction at 2 Gy for 4 cell lines: (A) combined treatment with 100-Hz modulated RFF and (B) combined treatment with 1000-Hz modulated RFF. Dashed lines represent the 95% confidence interval
99

LIST OF ABBREVIATIONS

α :	linear coefficient of inactivation after X-ray irradiation.
β :	quadratic coefficient of inactivation after X-ray irradiation.
AEF:	alternating electric fields.
Bax:	Bcl-2-associated X protein.
Bcl-2:	B-cell lymphoma-2.
BNI:	binucleation index.
c-jun:	protein encoded by the JUN gene in humans.
CO ₂ :	carbon dioxide.
\bar{D} :	mean inactivation dose.
DMSO:	dimethyl sulfoxide.
DNA:	deoxyribonucleic acid.
EBV:	Epstein-Barr virus.
egr-1:	early growth response protein 1.
EMF:	electromagnetic field.
Flk-1:	gene encoding VEGF-A receptor.
Gadd45:	growth arrest and DNA-damage-inducible protein.
HBV:	hepatitis B virus.
HCV:	hepatitis C virus.
HHV-8:	human herpes virus 8.
HIV:	human immunodeficiency virus.
HPVs:	human papillomaviruses.
HTLV-1:	human T-lymphotropic virus-1.
KDR:	kinase insert domain receptor.
KRAS:	Kirsten rat sarcoma viral oncogene homolog.
LQ:	linear-quadratic.
MCV:	Merkel cell polyomavirus.

MEM:	minimum essential medium.
MF:	modifying factor.
MNF:	micronucleus frequency.
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
p21:	phosphoprotein 21.
p53:	phosphoprotein 53.
PBS:	phosphate buffered saline.
NAD(P)H:	nicotinamide adenine dinucleotide phosphate.
OD:	optical density.
PE:	plating efficiency.
RFF:	radiofrequency fields.
ROS:	reactive oxygen species.
SEM:	standard error of the mean.
SF:	surviving fraction.
SF ₂ :	surviving fraction at 2 Gy.
SF ₆ :	surviving fraction at 6 Gy.
VEGF:	vascular endothelial growth factor.
WAF1:	protein encoded by p21.

CHAPTER 1

1.1. Introduction and Rationale

Cancer is a major cause of human death worldwide (Bray et al., 2012). Cancer arises from normal tissues, based on genetic predisposition, persistent inflammation, environmental factors, life style, and ageing (Katoh and Katoh, 2006). These are the factors that can be manipulated and controlled to prevent cancer. Once someone has developed cancer, the cancer cells acquire malignant cell behaviour which may include loss of the ability to regulate proliferation, resistance to apoptosis, senescence, and metastasis, to name a few (Hanahan and Weinberg, 2000; Katoh and Katoh, 2006). The characteristics exhibited by cancer cells are important factors that must be considered when developing effective treatment regimens for cancer (Katoh and Katoh, 2006). An effective treatment regimen is one that can overcome or counteract the factors that prolong the lifespan of cancer cells.

It is very challenging to treat cancer as cancer cells are continuously changing, and becoming resistant to treatment (Carmeliet and Jain, 2011). Many treatment options also have a toxic effect on normal cells resulting in patients suffering adverse side-effects (Mundy et al., 2003). Different cancers respond differently to different types of treatment. Some of the options used to treat cancer are surgery, chemotherapy, biological therapy and radiation therapy. Surgery is invasive and may leave patients disfigured. Also, some cancer cells may be missed and can develop into another tumour, leading to emotional stress (Kirova et al., 1998; Donato et al., 2013). Chemotherapy, radiation therapy and biological therapy can exert negative effects on

patients, as they can be very toxic to normal cells, with some patients dying from the side-effects caused by the treatment, and not from the cancer.

Another challenge is the co-morbidity of cancer with other infections making it more difficult to manage. Several viruses, such as, human immunodeficiency virus (HIV), human papillomaviruses (HPVs), Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), human herpes virus 8 (HHV-8), human T-lymphotropic virus-1 (HTLV-1), and Merkel cell polyomavirus (MCV), are linked with cancer in humans (Parkin, 2006). These infections weaken the immune system, with radiation therapy and other treatment options further weakening the already compromised immune system. The co-morbidity of cancer and other infections calls for a need to develop non-invasive methods to increase the sensitivity of cancer cells to therapy.

Radiation therapy is one of the most commonly used treatment options for various cancers, however, different tumours respond differently to different radiation doses. Melanoma, sarcoma and prostate cancer are examples of cancers that have a lower α/β ratio, making them more resistant to lower radiation doses, but sensitive to higher doses of radiation (Hegemann et al., 2014). High radiation doses pose a greater risk of normal tissue toxicity and a risk of developing severe side-effects. Examples of such severe effects are shown in Figures 1.1 and 1.2.

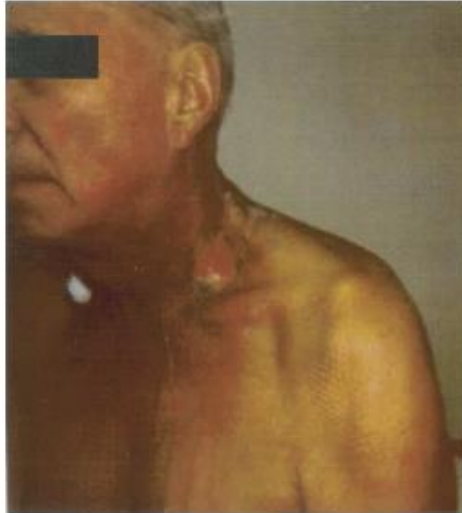


Figure 1.1: Radiotherapy-induced skin burns on a patient treated for carcinoma of the base of the tongue (from Lee et al., 2002).



Figure 1.2: Radiotherapy-induced late effects and ulceration (from Jacobson et al., 2017).

Considering the mentioned cancer treatment challenges there is a need to find regimens that eliminate cancers with minimal invasion and reduced normal tissue toxicity or side-effects. Radiofrequency waves are a possible candidate, in combination with radiation, to sensitise tumour cells to therapeutic doses of radiation.

For decades, electromagnetic fields (EMF) have been shown to exhibit diverse biological effects and therapeutic potential on their own or in combination with other treatment modalities (Simkó et al., 1998; Tofani et al., 2001; Czyz et al., 2004; Sarimov et al., 2005; Crocetti et al., 2013; Tofani, 2015; Lucia et al., 2016; Restrepo et al., 2016; Solek et al., 2017). The importance of radiation modifiers in radiotherapy, radiation protection, and biological dosimetry cannot be overemphasised. Therapeutic benefit has been demonstrated for the use of electromagnetic fields in cancer patients, where EMF treatment resulted in reduced disease progression, prolonged patient survival, and no significant side-effects (Kirson et al., 2007; Barbault et al., 2009; Verginadis et al., 2012). In fact, significant evidence exists suggesting that electromagnetic fields could potentially be the future of non-invasive and non-toxic therapy (Vadalà et al., 2016).

Although the mechanisms by which EMF interact with cells are not well understood, many studies have shown that electromagnetic fields of a wide range of frequencies can influence multiple cellular processes, such as cell proliferation, differentiation, cell cycle, apoptosis, DNA replication, production of reactive oxygen species, and protein/gene expression (Simkó et al., 1998; Tofani et al., 2001; Czyz et al., 2004; Sarimov et al., 2005; Crocetti et al., 2013; Tofani, 2015; Lucia et al., 2016; Restrepo et al., 2016; Solek et al., 2017). The multiplicity of effects of EMF extends to their capacity to stimulate the immune system (Walleczek, 1992), which may in turn mediate cellular responses to therapeutic interventions. Additionally, it has been suggested that some EMF, especially at high frequencies, can disrupt nervous system function and result in neurodegenerative disorders like autism (Ahuja et al., 2013), while others of lower frequencies stimulate damaged tissue recovery (Palti, 1966;

Bassett et al., 1981; Bassett, 1985; Polk, 1995; Markov and Colbert, 2001; Harden et al., 2007).

Although it was suggested two decades ago that EMF can enhance the effects of ionising radiation (Miyakoshi et al., 1999; Ding et al., 2000; Artacho-Cordón et al., 2013 for review), there is still a paucity of studies on the combined biological effects of EMF and ionising radiation. In a recent study by our group, no cytotoxic effects were observed in fibroblasts and melanoma cells, when cells were exposed to 27.125 MHz fields that were amplitude-modulated at 100 or 1000 Hz alone (Chinhengo et al., 2018). However, it was demonstrated that electromagnetic fields have the desirable radiosensitising and radioprotective effects on tumour (melanoma) and normal (fibroblasts) cells, respectively (Chinhengo et al., 2018). It was further shown that EMF may significantly reduce the total radiation dose during radiotherapy and minimise normal tissue toxicity without compromising on tumour control (Chinhengo et al., 2019). The diversity of effects, or lack thereof, is likely due to the wide range of frequencies, types of electromagnetic fields (electric, magnetic, or radio), and cellular systems used.

Given that radiofrequency waves show potential to act as radiosensitisers and radioprotectors, there is a need to understand the mechanisms underlying their modulatory effects. This may assist in designing patient- and tumour-specific therapeutic approaches that can lead to more effective cancer management.

1.2. Literature Review

1.2.1. Radiation Therapy Overview

Radiation therapy is one of the widely used cancer treatment options in the world (Liauw et al., 2013; Guadagnolo et al., 2013; Perez and Mutic, 2013; Hur and Yoon, 2017). Radiation induces cell death by direct or indirect damage to the DNA as illustrated in Figure 1.3. When radiation interacts with DNA, it causes single strand and double strand breaks. These breaks, if not efficiently repaired, obstruct the ability of the cells to divide and proliferate further. Double strand breaks are more responsible for cell death than the single strand breaks, as they are more difficult to repair (Jackson and Bartek, 2009; Baskar et al., 2012).

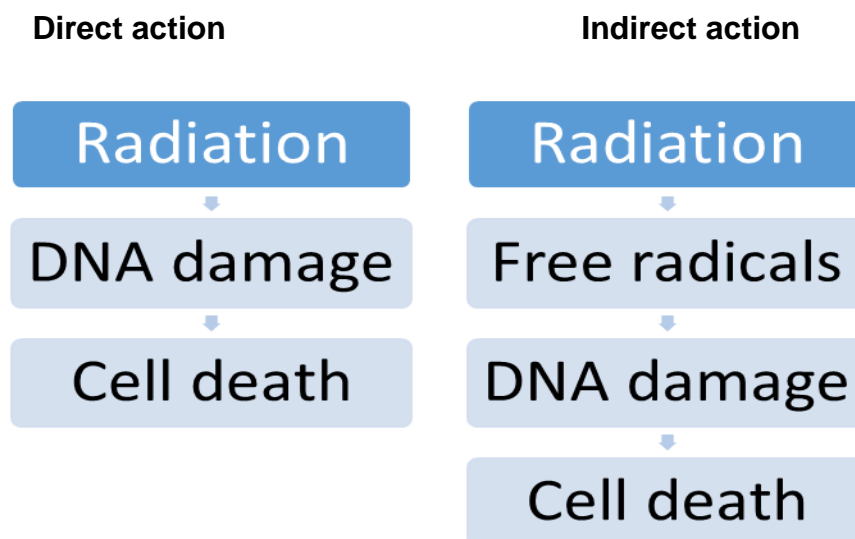


Figure 1.3: Radiation interacts with cellular DNA directly or indirectly (adapted from Basker et al., 2012).

Cells affected by radiation may die via various means, including apoptosis and necrosis. Ionising radiation also damages the cell membrane triggering pathways that are important in apoptosis and may lead to cell death, as illustrated in Figure 1.4 (Cohen-Jonathan et al., 1999).

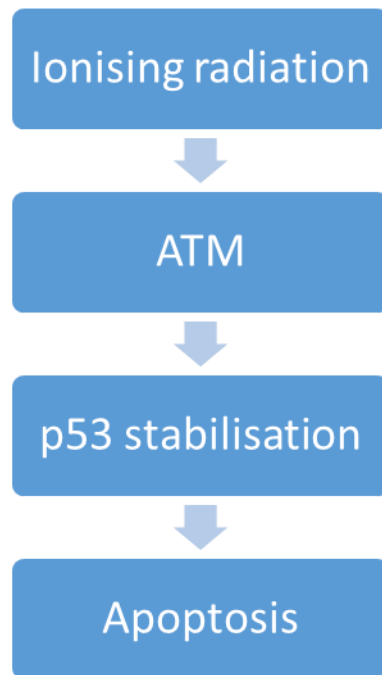


Figure 1.4: Radiation damage triggers a signal that is relayed from ATM to p53, leading to cell death (adapted from Cohen-Jonathan et al., 1999).

The dying tumour cells may also aid in tumour control by releasing cellular signals that boost antitumour immunity. A phenomenon called the bystander effect has been reported where dying irradiated tumour cells release signals that trigger neighbouring unirradiated tumour cells to die (Hur and Yoon, 2017; Peng et al., 2017). Radiation therapy does not only kill cancer cells, but also activates the immune system against future proliferation of cancer cells. This immune response is, however, limited and may not be able to completely control tumours.

The delivery of radiation therapy has improved significantly over time in terms of normal tissue sparing, but there is still damage to healthy tissue surrounding the tumour, with resultant adverse side-effects in patients. Radiotherapy can be delivered to patients in several small doses over the course of several weeks (hyperfractionation), or in large doses given over a shorter space of time (hypofractionation). In light of reported findings that exposure of cells to low radiation doses may blunt the p53 response, leading to a radioadaptive response (Takahashi, 2002), it may be prudent to use hypofractionated radiation therapy to effectively kill tumour cells without inducing a radioresistant response (Barlow et al., 2016). *In vitro*, hypofractionation treatment has been found to be more effective at killing tumour cells, leading to a delay in tumour outgrowth and improved cell survival rates, when compared to hyperfractionation. Hypofractionation may, therefore, be more beneficial in tumour control (Barlow et al., 2016).

Hypofractionated external beam radiotherapy has gained increasing popularity for cancers that have a lower α/β ratio, and are thus relatively more resistant to lower radiation doses but more sensitive to higher doses. For example, the treatment of melanoma, sarcoma, and prostate cancer is better when fractional radiation doses higher than the conventional 2-Gy are used. There are advantages to using hypofractionation over hyperfractionation, such as saving on treatment time and medical resources, making it more convenient for the patients (Hegemann et al., 2014). Hypofractionated radiation therapy is given over a shorter period than standard radiation therapy. It is becoming the standard of care for lung, breast, and prostate cancer patients. The main challenge of hypofractionation is normal tissue toxicity, which can be overcome if a modifying agent is used in combination with radiation. The

best modifying agent is one that sensitises the tumour whilst protecting the normal tissue. EMF have been found to sensitise cancer cells to radiation whilst protecting normal cells (Sylver, 2009; Crocetti et al., 2013; Chinhengo et al., 2018, 2019), suggesting that EMF can be a useful option as a radiomodulator in hypofractionated radiotherapy; and in hyperfractionated radiotherapy to sensitise cancer cells and possibly prevent a radioadaptive response.

1.2.2. Targeted Therapy

Targeted therapy is a cancer treatment that targets the cancer's specific genes, proteins, or the tissue environment that contributes to cancer growth and survival. Targeted therapy is designed to attack defects found in cancer cells but not in normal cells. There are several types of targeted therapy e.g. Monoclonal antibodies, which send toxic substances directly to cancer cells, and small-molecule drugs, which block the process that helps cancer cells multiply and spread e.g. angiogenesis inhibitors. The mode of action for targeted therapy drugs is to block or turn off signals that tell cancer cells to grow and divide, thus keeping cells from living longer than normal. The same targeted treatment will not work for all tumours, since not all tumours have the same targets. It is important to know the status of the targeted gene in order to use targeted therapy effectively e.g. cetuximab which targets KRAS will not be effective in treating a cancer with a defective KRAS gene (Nakadate et al., 2014)

Czyz and colleagues observed an upregulation of c-jun, p21 and egr-1 mRNA levels in p53-deficient cells, but not in p53 wild-type cells, when cells were exposed to a 1.71 GHz field (Czyz et al., 2004). C-jun and p21 regulate cell cycle progression. EMF

exposure has been shown to increase the expression levels of the VEGF receptor, KDR/FIk-1, in normal human umbilical vein endothelial cells (Delle Monache et al., 2008). Therapeutic targeting of VEGF has been tested and show positive results (Rini and Small, 2005). Since EMF cause upregulation and downregulation of certain genes and proteins they can be used to strategically upregulate or downregulate genes that control survival pathways in cancer cells. With further investigation, EMF can be useful in targeted therapy.

1.2.3. Combination Therapy

Combination therapy is a treatment option that combines two or more therapeutic agents (Mokhtari et al., 2017). Different treatment agents affect cancer cells at different stages of the cell cycle thus using more than one agent means more cells in the population are targeted, increasing the chances of eliminating cancer cells. Since the 1970's, combination chemotherapy has been used, but, the use of more than one drug at a time poses a risk of drug interactions which may negatively affect the patient. Combination chemotherapy may be effective with some cancers whilst single drug chemotherapy works better with other cancers. Combination therapy is advantageous in that it decreases the chance of a tumour developing resistance, for it addresses several targets at the same time, and since tumour cells vary from each other in heterogeneity, some cells may respond to one treatment agent whilst some respond to the other, and this may lead to the use of lower doses of drug or radiation.

The effectiveness of radiation therapy is influenced by many factors, such as the position of tumour, tumour progression and the size of the tumour. Other factors and/or

substances have been found to influence radiation therapy and so have been employed in combination therapy to further sensitise cancer cells to radiation. Some substances have been found to protect normal cells against the effects of radiation whilst making the tumour cells more sensitive to the same radiation dose.

Most cancers have defective housekeeping and tumour suppressor genes, such as, those encoding the heat shock protein 90 (*Hsp90*) and p53. These genes, when unaltered or not inactivated, are required for the maintenance of basic cellular function, and are expressed in all cells of an organism under normal and patho-physiological conditions (Levine et al., 1991; Barrot and Haystead, 2013). A defective p53 gene in cancer cells (e.g. the melanoma cell line, MeWo) may allow cells to proliferate, even after they have sustained radiation damage. Thus, there is a need to use substances, in combination with radiation, that may activate genes downstream of the defective gene so that the damaged cells can be forced to stop proliferating, through apoptosis or other gene-regulated means. A good agent to use in combination with radiation is one that influences the biochemical processes and gene expressions triggered by radiation, leading to improved tumour control. Recent work has shown that extremely low frequency electromagnetic fields can significantly enhance the cytotoxic effects of temozolomide, a widely-used chemotherapeutic agent, in glioblastoma cells (Akbarnejad et al., 2017).

1.2.4. The Co-morbidity of Cancer and Other Infections, and the Possible Influence of EMF on the Management of Such Cancers

Studies have demonstrated that infectious pathogens are associated with up to one-sixth of cancers worldwide (Parkin, 2006; de Martel et al., 2012). Infections with certain viruses, bacteria, and parasites are strong risk factors for specific cancers.

The co-morbidity of cancer with other infections makes it more difficult to manage. Several viruses, such as, human immunodeficiency virus (HIV), human papillomaviruses (HPVs), Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), human herpes virus 8 (HHV-8), human T-lymphotropic virus-1 (HTLV-1), and Merkel cell polyomavirus (MCV), are linked with cancer in humans (Parkin, 2006). Examples of HHV-8 associated Kaposi's sarcoma and HPV-mediated genital warts associated with cervical cancer are illustrated in Figures 1.5 and 1.6, respectively.



Figure 1.5: HHV-8 associated Kaposi's Sarcoma (National Cancer Institute visuals online, 2001; <https://visualsonline.cancer.gov/details.cfm?imageid=2168>).



Figure 1.6: HPV-mediated genital warts associated with cervical cancer (Feigwarzen Bilder; <https://www.warzen-hilfe.com/feigwarzen-welcher-arzt/>)

These infections weaken the immune system, and radiation therapy further weakens the already compromised immune system. The co-morbidity of cancer and other infections calls for a need to develop non-invasive methods to sensitise cancer cells to therapeutic treatment doses. The best treatment options would be ones that target the cancer cells and the associated bacteria, viruses or parasites.

Electromagnetic fields (EMF) have been reported to activate the immune system and sensitise cancer cells to radiation, so they may be used in combination with radiation to improve treatment of cancers associated with other infections. Inhan-Garip and colleagues found that EMF induce a decreased growth rate in some bacteria (Inhan-Garip et al., 2011) and its use in combination with radiation may, therefore, be useful in treating cancers associated with other infections. Exposure of oncogenic viruses to extremely low frequency electromagnetic fields has been shown to lead to defective viral progeny (Pica et al., 2006).

The association of cancer with other infections makes it more challenging to treat effectively as other infections further weaken the immune system and sometimes intensify the side-effects posed by radiation therapy. There is, therefore, the need to find agents that can sensitise the cancer cells to radiation whilst protecting normal tissue, thus improving tumour control without further compromising the normal tissue. EMF may be a good modifying agent to use in combination with radiation for the treatment of cancers associated with other infections.

1.2.5. Effects of Electromagnetic Fields

For over half a century, there has been a growing interest in the investigation of extremely low, through medium, to high frequency electromagnetic fields for their potential application in the clinic. These fields may be classified as non-thermal non-ionising radiation, present as magnetic, electric, or radiofrequency waves, and have been shown to exhibit a wide range of effects in biological systems. The extent of the attributes of electromagnetic fields has led to suggestions that they have a high potential of becoming the future of holistic medicine, with the capacity of curing cancer and other ailments (Sylver, 2009; Purnell and Whitt, 2016).

1.2.5.1. Electric Fields

Alternating electric fields (AEF) have shown a wide range of effects on living tissues, in a frequency-dependent manner. At very low frequencies (under 1 kHz), AEF can stimulate excitable tissues and have been used for nerve, muscle, and heart stimulation to promote regeneration (Palti, 1966; Polk, 1995; Markov and Colbert,

2001). Electric fields also stimulate osteoblast proliferation and could enhance the efficacy of orthopaedic implants (Ercan and Webster, 2008).

Although several non-thermal cellular effects of AEF have been observed (Zimmerman et al., 1981; Holzapfel et al., 1982; Pawlowski et al., 1993), a number of *in vitro* and *in vivo* studies have illustrated the antiproliferative and anticancer effects of alternating electric fields (Kirson et al., 2004; Kirson et al., 2007). These interesting results led to the implementation of such approaches in humans, and initial results on a number of tumour entities/sites were encouraging (Kirson et al., 2007; Salzberg et al., 2008).

Furthermore, studies involving low-intensity, intermediate frequency (100-300 kHz) alternating electric fields have clearly documented that these antiproliferative effects are mediated by processes like cell cycle arrest and mitotic disruption (Gera et al., 2015; Giladi et al., 2015). A significant level of evidence exists of therapeutic responses in patients with cancer, demonstrating that electric field treatment results in a retardation of disease progression and extended patient survival, with little or no obvious side-effects (Kirson et al., 2007; Verginadis et al., 2012; Davies et al., 2013; Mrugala et al., 2017; Benson, 2018; Burri et al., 2018; Magouliotis et al., 2018).

Electric fields have been extensively shown to enhance the therapeutic efficacy of other treatment modalities. Mild electrical stimulation, when combined with hyperthermia, has been reported to reduce insulin resistance and enhance fat metabolism in diabetic mice (Morino et al., 2008). A strong synergy in colorectal cancer cell killing in a mouse xenograft model was demonstrated, when tumours were treated

with an electric field and hyperthermia (Andocs et al., 2009). Electric field treatment enhances the cytotoxic effects of a number of chemotherapy drugs in *in vitro* cell cultures, animal tumour models (e.g. recurrent glioblastoma multiforme, hepatocellular carcinomas, breast carcinomas), and clinical trials (Janigro et al., 2006; Kirson et al., 2007; Salzberg et al., 2008; Kirson et al., 2009a). O'Connell and colleagues demonstrated the capacity of combined electric field and bevacizumab treatment to significantly stabilise grade IV astrocytoma in a paediatric patient (O'Connell et al., 2017). Kesari and colleagues also reported a prolonged overall survival in glioblastoma patients when they were treated with a combination of electric fields and temozolomide after a first recurrence (Kesari et al., 2017). An extension of overall survival of more than seven years has been documented for patients with primary and recurrent glioblastoma multiforme who received electric field therapy (Rulseh et al., 2012). Several other studies have demonstrated the efficacy of electric fields, administered alone or in combination with chemotherapy drugs, to inactivate cancer cells, improve tumour control, prolong patient survival, and maintain quality of life (Schneiderman et al., 2010; Stupp et al., 2012, 2015, 2017; Vergote et al., 2018; Toms et al., 2019).

In addition to preventing tumour growth, electric fields have been shown to inhibit tumour cell migration, invasion, and metastasis (Kirson et al., 2009b; Kim et al., 2016). They also exhibit antimicrobial effects (Giladi et al., 2008, 2010; Gabi et al., 2011; Freebairn et al., 2013; Shawki and Gaballah, 2015), and may be an effective modality for treating infections. Currently, application of electric fields in the clinical setting is expanding, especially in the domain of cancers of the central nervous system.

1.2.5.2. Magnetic Fields

Low-frequency pulsed magnetic fields have also shown a markedly wide range of effects in biologic systems, and have been useful in clinical applications. These fields have been known to stimulate various tissues to enhance repair, as in the cases of increased bone and cartilage growth, accelerate fracture healing, and relief of low back pain; and have even been used to treat depression (Bassett et al., 1981; Bassett, 1985; Markov and Colbert, 2001; Harden et al., 2007; Parate et al., 2017; Madduri et al., 2018). Magnetic fields have also been used to successfully treat wounds (Cheing et al., 2014; Haghnegahdar et al., 2014; Cañedo-Dorantes et al., 2015; Zahedi and Yadollahpour, 2016; Madduri et al., 2018; Kwan et al., 2019). The mechanisms underlying the efficacy of magnetic fields in improving wound healing include enhanced fibroblast polarisation and migration (Purnell and Skrinjar, 2016), increased deposition of collagen fibre (Choi et al., 2016), and stimulation of cell proliferation (Cheng et al., 2017). Magnetic field treatment has also been found to enhance nerve regeneration (Beck-Broichsitter et al., 2014), postnatal neovascularisation (Li et al., 2015), and human erythrocyte health (Purnell et al., 2018a). The benefit of magnetic fields in restoring cardiac function has also been documented (Hao et al., 2014; Ma et al., 2016).

Low level electromagnetic energies, *akin* to those of magnetic fields, exert negative influences on cancer cells, but not their normal counterparts (Crocetti et al., 2013; Purnell et al., 2018b; Purnell, 2019). The antitumour effects of magnetic fields have been extensively demonstrated in a number of tumour entities/sites (Ronchetto et al., 2004; Verginadis et al., 2012; Sengupta and Balla, 2018). These fields have long been

known to stimulate the activity of immune cells (Walleczek, 1992; Baranowska et al., 2018; Rosado et al., 2018; Mahaki et al., 2019), which may subsequently enhance antitumour immunity in cancer patients.

A growing number of studies have attributed the anti-proliferative effects of magnetic fields on cancer cell changes in metabolic activity, differentiation, cell cycle progression, and the profiles of apoptosis, DNA replication, production of reactive oxygen species, and protein/gene expression (Lai and Singh, 1997; Simkó et al., 1998; Tofani et al., 2001; Sarimov et al., 2005; Morabito et al., 2010; Kovacic and Somanathan, 2010; Crocetti et al., 2013; Tofani, 2015; Lucia et al., 2016; Restrepo et al., 2016; Solek et al., 2017; Novoselova et al., 2019). Magnetic fields have been shown to cause G1 phase arrest by activating the ATM-Chk2-p21 pathway (Huang et al., 2014). Solek and colleagues also found that magnetic fields can induce the p53/p21-mediated apoptotic signalling pathway and cell cycle arrest in mouse spermatogenic cells *in vitro*, and concluded that these fields may affect fertility (Solek et al., 2017).

In addition, chemotherapy drug cytotoxicity enhancing effects of magnetic fields have been widely demonstrated in cell cultures, in *in vivo* models, and patients (Salvatore et al., 2003; Baharara et al., 2016; Sengupta and Balla, 2018). Although there has been a growing interest in research involving electromagnetic fields, only a handful of studies have reported of the biological effects of magnetic fields with ionising radiation (Miyakoshi et al., 1999; Ding et al., 2000; Artacho-Cordón et al., 2013).

1.2.5.3. Radiofrequency Fields

The biological effects of radiofrequency fields are varied, and may be categorised as genotoxic (e.g. chromosomal aberration, micronucleus formation) and non-genotoxic (e.g. gene expression, cell proliferation, reactive oxygen species) (Miyakoshi, 2013). For medical and research purposes, radiofrequencies are generated by amplitude modulating a carrier frequency of ~27.12 MHz. The antitumour effects of radiofrequency fields (RFF) have been attributed to a unique phenomenon of the existence of tumour-specific modulating frequencies, whereby fields modulated by certain frequencies only exert their effects on particular tumours (Elson, 1995; Zimmerman et al., 2013; Jimenez et al., 2018). Radiofrequency tumour ablation, mediated by this phenomenon, is correlated with the dielectric property of the tumour in question and, thus, its water content (Chou, 1995; 2007). Treatment of patients with advanced hepatocellular carcinoma with modulating frequencies ranging from 100 Hz to 21 kHz yielded positive results (Costa et al., 2011). Barbault and colleagues treated 163 patients for 15 cancers with modulating frequencies of 100 Hz – 114 kHz and noted that the most common specific frequencies for breast cancer, hepatocellular carcinoma, prostate cancer and pancreatic cancer were 1873.477 Hz, 2221.323 Hz, 6350.333 Hz, and 10456.383 Hz, respectively (Barbault et al., 2009). Frequency-dependent antiproliferative effects were also demonstrated in hepatocellular carcinoma and breast cancer cells, but not in normal cells (Zimmerman et al., 2012).

The selectivity of radiofrequencies in negatively affecting only malignant cells could be harnessed for more effective cancer management. However, unlike for electric and

magnetic fields, studies investigating the combined effects of radiofrequency fields and chemotherapeutic drugs or ionising radiation are virtually non-existent.

A couple of studies performed by our group, using fibroblasts and melanoma cells show that electromagnetic fields can act either as radiosensitisers or radioprotectors (Chinhengo et al., 2018; 2019). An insight into the factors that influence these modifying effects could have significant implications in radiotherapy and radiation protection settings.

DNA damage by ionising radiation induces the gene expression of p53-target molecules, involving WAF1, Gadd45, Bax and p53. Most tumour cells, however, have a mutant or defective p53 gene and this could allow abnormal and damaged cells to proliferate. Other genes downstream of p53 can be manipulated to regulate the cell cycle and suppress tumour progression. Czyz and colleagues found that the exposure of p53 deficient cells to high frequency radio waves (1.71 GHz) induced an upregulation of egr-1 (an early growth response gene), a phenomenon not seen in p53 wild-type cells, and suggested that loss of p53 function may affect the sensitivity of cells to radiofrequency exposure (Czyz et al., 2004). It is, therefore, possible that p53 status plays a key role in the manner by which radiofrequencies modulate cellular response to ionising radiation.

Radiofrequency fields may cause an increase in endogenous cellular factors that result in elevated levels of reactive oxygen species (ROS) and DNA damage (Phillips et al., 2009). This may be due to increased mitochondrial membrane permeability, pore formation, and increased nicotinamide adenine dinucleotide phosphate

(NAD(P)H) content (Dumas et al., 2009). An increase in NAD(P)H may lead to an increase in the formation of ROS, which is mediated by NAD(P)H oxidase. NAD(P)H oxidase reduces oxygen to the reactive oxygen species, oxygen radical and hydrogen peroxide. The primary and sole function of NAD(P)H oxidase is ROS production, so the absence of NAD(P)H prevents formation of reactive oxygen species (Raza et al., 2017). NAD(P)H is a coenzyme for many enzymes in the cell. Other functions of NAD(P)H include the detoxification of xenobiotics, participation in amino acid metabolism, as a requirement in immune functions, like phagocytosis, and synthesis of fatty acids. Therefore, a depletion of NAD(P)H may prevent the proper functioning of cells.

To maintain ROS homeostasis and avoid cell death, cancer cells increase their antioxidant capacity. This altered redox environment of cancer cells, compared with normal cells, may increase their susceptibility to ROS-manipulation therapies (Reczek and Chandel, 2017). Cancer cells require a certain level of ROS, above or below which cell death is induced or promoted. This biochemical difference between cancer and normal cells can be exploited to develop and employ therapeutic agents to preferentially target cancer cells (Raza et al., 2017). It would, therefore, be interesting to interrogate the role of reactive oxygen species in mediating radiomodulatory effects of radiofrequency fields.

Iron content in cells determines the production of free radicals. As cancer cells have higher iron content, they should be expected to be more responsive than normal cells to magnetic fields induced by radiofrequency fields. The action of induced magnetic fields in cells is mediated by the Fenton effect, as demonstrated in Figure 1.7.

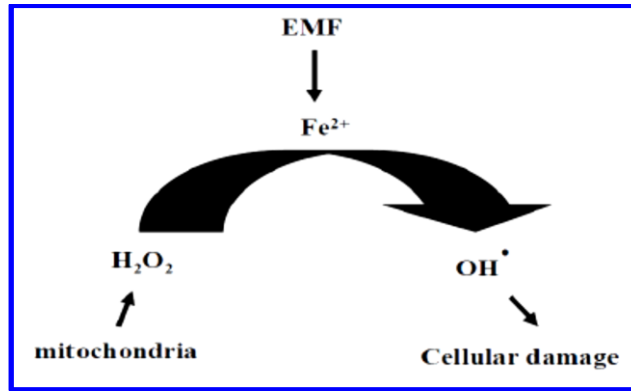


Figure 1.7: The Fenton reaction: conversion of hydrogen peroxide (H₂O₂) into a highly reactive hydroxyl radical (OH[•]) in the presence of iron (Phillips et al., 2008).

Taking the abovementioned diverse effects of EMF into account, it can be reasoned that EMF would play a key role in the manner by which ionising radiation affects biological systems. The main objectives of this study were to: (1) further interrogate the finding that low-medium radiofrequency waves can act both as radiosensitisers and radioprotectors (Chinhengo et al., 2018, 2019), and (2) identify potential mechanisms underlying such phenomena, using an expanded panel of established cancer and normal cell lines. It is anticipated that the findings of this study will form the basis for future studies on human specimens, such as peripheral blood lymphocytes, especially if they can be extrapolated to doses of relevance to radiation protection and biological dosimetry. The study should contribute significantly toward a more reliable interpretation of the relationship between radiation absorbed dose and effect, as well as, inform practitioners in radiotherapy, radiation and environmental protection, occupational health, and biological dosimetry on delivery of more efficient services.

1.3. Problem Statement

There are many challenges encountered in cancer therapy, the major ones being resistance to treatment and normal tissue toxicity. Melanoma, sarcoma and prostate cancer are some of the superficial cancers that have a lower α/β ratio. These cancers are best treated using hypofractionation (i.e. higher fractional doses of radiation), as they do not respond well to low doses per fraction. Hypofractionation, however, leads to high normal tissue toxicity which the patient may not easily recover from. There is an urgent need to develop non-invasive methods, to further sensitise cancer cells (or tumours) to therapeutic doses of ionising radiation. Such procedures may induce radioprotection to the normal tissue while further sensitising the tumour to radiation, thus achieving better tumour control and reducing toxicity to normal tissue. On the other hand, interpretation of the effects of low doses of radiation can be complicated by interaction between ionising radiation and ambient factors, such as those involved when employing potentially non-invasive approaches with the aim of sensitising cancer cells. Identifying mechanisms underlying such interactions would have significant ramifications for radiation risk assessment (radiation protection).

1.4. Research Question

Do radiofrequency electromagnetic waves modulate the radiosensitivity of cancer and normal cells *in vitro*? If so, are there identifiable mechanisms that mediate such modulatory effects?

1.5. Hypothesis

It is hypothesised that *in vitro* exposure of cancer cells to radiofrequency electromagnetic waves can preferentially increase their radiosensitivity; and that this phenomenon is mediated by modifications in identifiable cellular features.

1.6. Aims and Objectives

This study aimed to assess the potential therapeutic benefit of combining radiofrequency fields (RFF) with radiation therapy in an *in vitro* setting and to identify factors mediating such benefits, using human lung fibroblasts, prostate cancer cells, and melanoma cells.

To achieve these specific aims, the specific study objectives are as follows:

1. To identify the radiofrequency and X-ray combinations that significantly increase the radiosensitivity of melanoma and prostate cancer cells, relative to normal lung fibroblasts.
2. To monitor superoxide dismutase (SOD) activity following exposure to radiofrequency waves and X-rays, in order to evaluate how oxidative stress is involved in the modulation of radiosensitivity by RFF.
3. To evaluate the effect of combined RFF and X-ray exposure on metabolic activity, in order to determine how changes in metabolic activity are related to the radiomodulatory effects of radiofrequency waves.
4. To evaluate the effect of RFF exposure on DNA damage responses, based on micronucleus yield.

1.7. Delineations

This study used the PERL M⁺ oscillator amplifier (Resonant Light Technology, Courtenay, Canada; Serial #: PM 171116) to produce 27.125 MHz fields, square-wave amplitude-modulated at 100, 1000, 2000 or 4000 Hz, to evaluate the effect of radiofrequency fields in cell cultures. The resulting radiofrequencies were broadcast via an argon plasma ray tube, acting as an antenna, onto the cells. Four cell lines were used in this study: a p53 mutant melanoma cell line, MeWo; a p53 wild-type melanoma cell line, Be11; a p53 mutant prostate cancer cell line, DU145; and a p53 wild-type normal lung fibroblast cell line, L132.

The research variables determined in this study were intrinsic cellular radiosensitivity, the radiomodulatory effects of radiofrequency fields and treatment-induced changes in metabolic activity, reactive oxygen species formation, and DNA damage and repair. The colony forming assay was used to determine intrinsic radiosensitivity and radiomodulation by means of radiofrequency fields. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay was used to measure treatment-induced changes in metabolic activity. The micronucleus assay was used to measure the effect of treatment on DNA damage and repair. The SOD assay was used as a measure of ROS levels. Modifying factors were derived from all the assays to determine how the cells responded to the treatment. These research variables were deemed enough to prove the research hypothesis.

1.8. Limitations

- The melanoma and prostate cancer cell lines were compared to a normal lung cell line instead of corresponding normal prostate and skin cell lines for true representation.
- Due to financial constraints, the SOD assay was only performed using one frequency of radiofrequency fields.
- The effect of treatment on protein and / or gene expression was not performed due to time and financial constraints.

Two of the cell lines (DU145 and MeWo) were derived from metastatic lesions, and it has been shown that metastatic cancer has increased mutation burden compared to primary cancer, and that immune related gene expression is higher in metastatic cancer cell lines than in cell lines derived from non-metastatic cancer (Liu et al., 2019). A metastatic cell line may not be a true representative of the primary tumour.

CHAPTER 2

2. Materials and Methods

2.1. Study Location and Ethical Consideration

All experiments were performed in the Division of Radiobiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg. The study was approved by the Health Research Ethics Committee (HREC) of the Faculty of Medicine and Health Sciences, Stellenbosch University, South Africa (HREC Reference #: S17/10/207; Appendices A & B).

2.2. Cell Lines and Culture Maintenance

Four cell lines (2 melanoma, 1 prostate cancer, 1 lung fibroblast; 2 p53 mutant, 2 p53 wild-type) were used in this study and are detailed below:

MeWo

The MeWo cell line (ATCC® Number: HTB-65™) is a p53 mutant, adherent, human malignant melanoma cell line derived from a metastatic lymph node, and was kindly provided by Profs F. Zölzer and C. Streffer (University of Essen, Germany). The cells were routinely cultivated as monolayers in 75 cm² flasks in minimum essential medium (MEM), supplemented with 20% foetal bovine serum (FBS) and 1% penicillin-streptomycin, and incubated at 37°C in a humidified atmosphere (95% air, 5% CO₂). Cells were used for experiments upon reaching 80-90% confluence (passage numbers: 17-25).

Be11

The BE11 cell line is a p53 wild-type, human melanoma cell line, and was also kindly provided by Profs F. Zölzer and C. Streffer (University of Essen, Germany). The cells were routinely cultivated as monolayers in 75 cm² flasks in MEM, supplemented with 10% FBS and 1% penicillin-streptomycin, and incubated at 37°C in a humidified atmosphere (95% air, 5% CO₂). Cells were used for experiments upon reaching 80-90% confluence (passage numbers: 12-21).

DU145

The human prostate cancer cell line, DU145 (ATCC® Number: HTB-81D™), is a p53 mutant, adherent cell line derived from a metastatic lesion of the central nervous system (Stone et al., 1978), and was a gift from Prof P. Bouic (Synexa Life Sciences, Montague Gardens, South Africa). The cells were also cultivated as monolayers in 75 cm² flasks in MEM, supplemented with 10% FBS and 1% penicillin-streptomycin, and incubated at 37°C in a humidified atmosphere (95% air, 5% CO₂). Cells were used for experiments upon reaching 80-90% confluence (passage numbers: 16-18).

L132

The human normal lung epithelial cell line, L132 (ATCC® Number: CCL-5™), is a p53 wild-type cell line, and was a gift from Dr T. Robson (University of Ulster, UK). L132 was used to represent normal tissue. The cells were routinely cultivated as monolayers in 75 cm² flasks in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS and 1% penicillin-streptomycin, and incubated at 37°C in a humidified atmosphere (95% air, 5% CO₂). Cells were used for experiments upon reaching 80-90% confluence (passage numbers: 24-30).

2.3. Preparation of Cell Cultures for Experiments

Exponentially growing cell cultures were trypsinised into single-cell suspensions and seeded in varying numbers into 25-cm² tissue culture flasks (for colony and superoxide dismutase activity assays), 35-mm plastic petri dishes (for micronucleus assay), or 96-well cell culture plates (for metabolic activity assay), dependent on the level of absorbed radiation dose, cell line, and experiment. The corresponding final volumes of culture medium were 10 ml, 2 ml, and 100 µl, respectively.

2.4. Irradiation of Cell Cultures

Appropriately prepared cell cultures were irradiated at room temperature (20°C) to doses up to 10 Gy, at a dose rate of 1.0 Gy/min, using a Precision MultiRad 160 X-irradiator (Precision X-Ray Inc., Branford, CT, USA), as shown in Figure 2.1. Sham-irradiated cultures (0 Gy) were left on the turntable of the running Precision X-ray irradiator for 2 min with the X-ray source turned off and were used as controls.

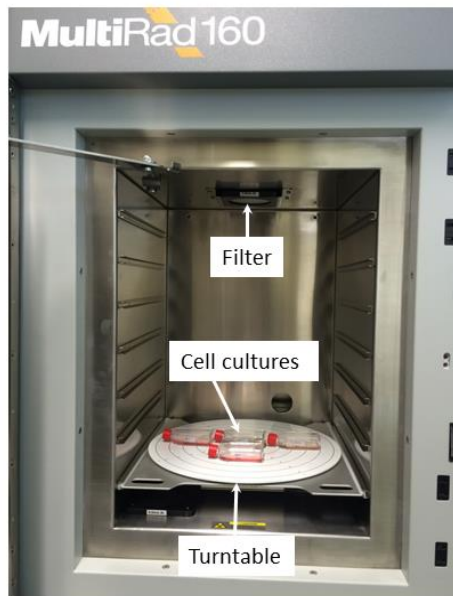


Figure 2.1: Photograph of the Precision MultiRad 160 X-ray irradiator (door opened) showing cell culture flasks on the turntable.

2.5. Radiofrequency Generation and Exposure

For cell culture exposure to radiofrequency fields, a PERL M⁺ oscillator amplifier (Resonant Light Technology, Courtenay, Canada; Serial #: PM 171116) was used to produce 27.125 MHz fields, square-wave amplitude-modulated at 100, 1000, 2000 or 4000 Hz, with a peak-to-peak amplitude of 5 V. The modulating frequencies were generated, using a ProGen II frequency generator with an output impedance of 50 Ω and a duty cycle of 50% (Resonant Light Technology, Courtenay, Canada; Serial #: PG 171211). The resulting radiofrequencies were then broadcast via an argon plasma ray tube (length: 29 cm, diameter: 2.55 cm, pressure: 20 Torr), acting as an antenna, onto the cells. For sham-RFF exposure (no radiofrequency field), unirradiated (0 Gy) cell cultures were placed under the plasma ray tube when turned off. The set-up is shown in Figure 2.2A. A maximum of 16 cell culture flasks, stacked in groups of four, could be exposed at a given time. As illustrated in Figure 2.2B, the volume occupied

by the cell culture layers had outside dimensions of 11 cm (Width: 2 flasks breadthwise) × 18 cm (Length: 2 flasks lengthwise) × 10 cm (Height: 4 flasks by height). The perpendicular distances from the axis of the plasma tube to the four cell culture planes were 19.0, 21.4, 23.8, and 27.0 cm. Each cell layer was covered with 10 ml of culture medium (medium depth: 3.5 mm).

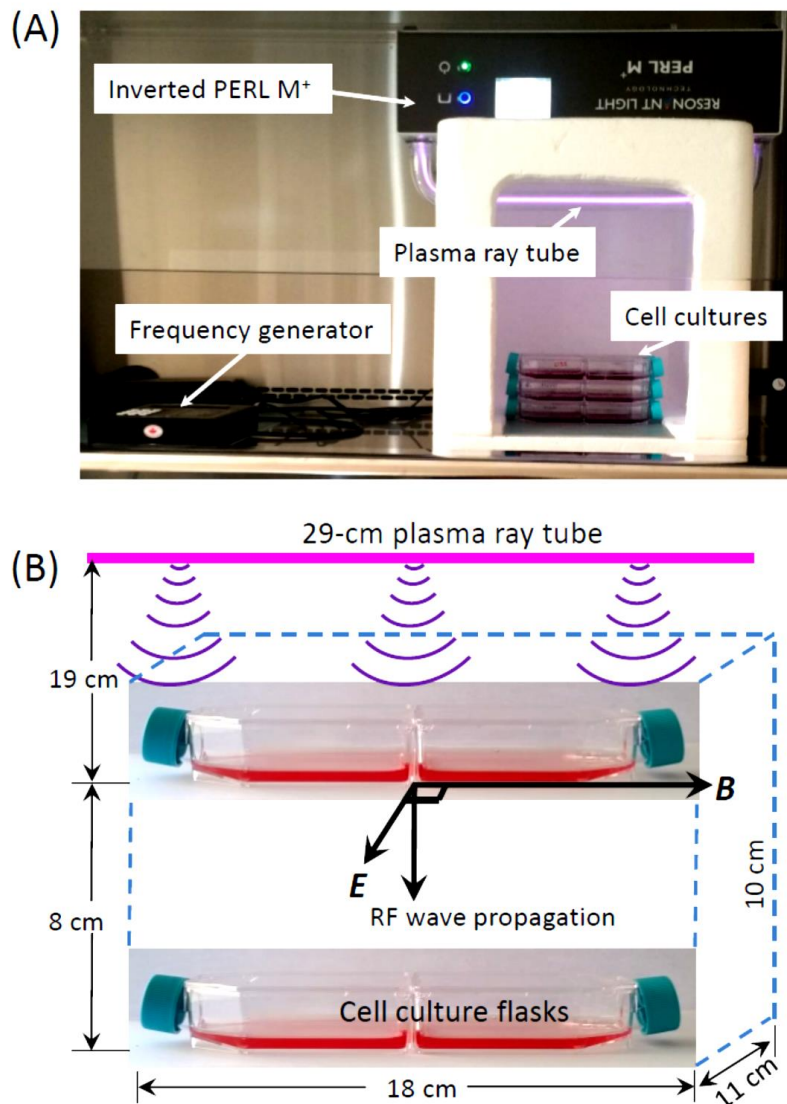


Figure 2.2: (A) Photograph of the electromagnetic field (EMF) exposure system, with the PERL M⁺ inverted on an appropriately cut styrofoam box. (B) A 2-dimensional schematic diagram showing the top and bottom cell culture planes of the 2×2×4 flask matrix. In the set-up, the plasma ray tube is centred horizontally above the cell culture flasks, such that the induced magnetic field (B) is parallel to the base of a flask and the induced electric field (E) in the culture medium is parallel to the width of the flask.

The induced electric fields in the cell cultures were estimated, using a large-loop H-Field probe (Beehive Electronics, California, USA; cat # 100C) coupled to a digital storage oscilloscope (Hantek Electronic Co. Ltd., Qingdao, China; Serial #: DSO5062BM). For this, the loop was positioned perpendicularly (in air) to the RF wave (Figure 2.2B), at the respective cell culture planes. During sham exposure (background), the electric field was found to be 3.7 V/m. The induced peak electric fields (E_{peak}) were measured in triplicate for each modulated frequency and perpendicular distance from the plasma ray tube. No significant frequency-dependent variation in the induced electric fields was observed, and the mean of all measurements at each plane was taken as the peak electric field at that location.

The induced peak electric fields (background subtracted) were then used to estimate the peak magnetic flux density (B , in T) from $E_{\text{peak}} = 2h\pi fB$ (Bassen et al., 1992); where f is the transmitted frequency (27.125×10^6 Hz), and $2h$ is the depth of the cell culture medium (0.0035 m). The induced current densities (J) were also estimated from the peak electric fields, according to the relation $J = \sigma E$, assuming a conductivity (σ) of 1.5 S/m for the cell culture medium (Bassen *et al.*, 1992). The estimated induced peak electric fields, magnetic flux density, and current density are presented in Table 2.1.

Table 2.1: Estimated peak electric field (E), magnetic flux density (B), and current density (J) induced at a distance (d) from the axis of a 29-cm plasma ray tube.

d (cm)	E (V/m)	B (μ T)	J (A/m ²)
19.0	6.69 ± 1.15	22.43	10.04
21.4	5.25 ± 0.69	17.60	7.88
23.8	3.24 ± 1.11	10.86	4.86
27.0	2.01 ± 0.51	6.74	3.02

2.6. Clonogenic Cell Survival Assay

Table 2.1 summarises the cell numbers that were seeded per dose point for each cell line. For each experiment and dose point, cell culture flasks were prepared in triplicate.

Table 2.2: Summary of cell numbers seeded at each radiation dose of X-rays delivered acutely.

Cell line	Dose (Gy)						
	0	1	2	4	6	8	10
MeWo	200	200	300	1000	3000	8000	15000
Be11	200	200	300	1000	2000	5000	10000
DU145	200	200	300	1000	2000	6000	10000
L132	200	200	300	1000	3000	7000	15000

All seeded cells were left to settle for 3 h after which they were irradiated with X-rays to the specified doses. The cell cultures were then incubated at 37°C in a humidified atmosphere (95% air, 5% CO₂) for 10 days (for DU145, Be11 and L132) and 15 days (for MeWo) for colony formation.

To terminate cultures, the growth media were decanted and colonies were washed with phosphate buffered saline, fixed in glacial acetic acid:methanol:water (1:1:8,v/v/v), stained in 0.01% amido black in fixative, washed in tap water, air-dried, and counted using a stereoscopic microscope (Nikon, Japan; Model #: SMZ-1B). Colonies containing at least 50 cells were deemed to have originated from single surviving cells and were scored. Cytotoxicity was assessed on the basis of a surviving fraction (SF) which was calculated from the relation:

$$SF = n_{col}(t) / \{ [n_{col}(u) / n_{cell}(u)] \times n_{cell}(t) \} \quad (2.1),$$

where $n_{col}(t)$ and $n_{col}(u)$ denote the number of colonies counted in treated and untreated samples, respectively. $n_{cell}(t)$ and $n_{cell}(u)$ are the number of cells seeded in treated and untreated cultures, respectively.

Three independent experiments were performed for each cell line. To generate clonogenic cell survival curves, the determined mean surviving fractions (SF) were fitted to the linear-quadratic (LQ) model of the form:

$$SF = \exp[-\alpha D - \beta D^2] \quad (2.2),$$

where α and β are the linear and quadratic coefficients, respectively, and D is the dose in Gy. Cellular radiosensitivity was expressed in terms of several indicators, namely, the surviving fraction at 2 Gy (SF_2), the surviving fraction at 6 Gy (SF_6), the mean inactivation dose (\bar{D}), and the α/β ratio. The SF_2 and SF_6 represent low and high dose sensitivity, respectively. The mean inactivation dose, which is the area under the survival-dose response curve plotted on a linear-linear scale, represents the sensitivity over low-high doses. The α/β ratio depicts both the steepness and curvature of a survival curve, and is the dose at which the linear and quadratic components of cell killing are equal.

2.7. Effect of Radiofrequency Fields on Plating Efficiency

To assess if the various radiofrequencies have any cytotoxic effect on the cells, plating efficiencies determined from unirradiated cultures with no exposure to RFF were compared with those from unirradiated cultures exposed to RFF, to obtain a plating efficiency modifying factor:

$$MF_{\text{plating efficiency}} = \frac{n_{\text{col}}(0 \text{ Gy})/n_{\text{cell}}(0 \text{ Gy})}{n_{\text{col}}(0 \text{ Gy}+\text{RFF})/n_{\text{cell}}(0 \text{ Gy}+\text{RFF})} \quad (2.3),$$

where n_{col} and n_{cell} are the number of colonies counted and cells seeded, respectively.

2.8. Radiomodulatory Effects of Radiofrequencies

To investigate the influence of RFF exposure on radiosensitivity, stock cell cultures from all 4 cell lines were trypsinised and cells seeded in numbers as listed in Table 2.1 per 25-cm² tissue culture flask and left to settle for 3 h. The cells were subsequently exposed to radiofrequency fields modulated at 100 and 1000 Hz for 30 min, as described in Section 2.5, 2 h prior to or following 1-10 Gy of X-ray irradiation (Section 2.4). Unirradiated cultures with and without RF field exposure were used as controls for RFF and X-ray treatment, respectively. The cell cultures were then processed for colonies to form. Surviving fractions were determined for three independent experiments for each radiation dose point and frequency, and corresponding survival curves were generated as per Equation 2.2. The modulatory effect of radiofrequency fields on radiosensitivity was expressed as a survival modifying factor (MF_{survival}), given

as the ratio of surviving fractions at a dose of X-rays (or the mean inactivation dose, \bar{D}) in the absence and presence of RFF:

$$MF_{survival} = \frac{SF(X-rays)}{SF(RFF+X-rays)} \text{ or } \frac{\bar{D}(X-rays)}{\bar{D}(RFF+X-rays)} \quad (2.4).$$

The criteria for inhibition, no effect, and enhancement of radiosensitivity by RFF are $MF < 1.0$, $MF = 1.0$, and $MF > 1.0$, respectively.

To test for potential radiomodulatory effects of fields modulated at higher frequencies, the procedure described here was repeated with fields modulated at 2000 and 4000 Hz when combined with 2 and 6 Gy. The corresponding surviving fractions were derived and compared with those when cell cultures were treated with X-rays alone, according to Equation 2.4.

2.9. Assessment of Metabolic Activity

Treatment-induced changes in cellular metabolic activity were measured, using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. For this, 100 000 cells in 100 μ l of medium were seeded per well into a 96-well cell culture plate (triplicate per sample), with detachable wells to enable selective treatment and reassembly. The cells were incubated for 2 hours to attach. Four wells were then used for each treatment option, namely, treatment with radiofrequency waves modulated by either 100 Hz or 1000 Hz, 2 h prior to or post 6 Gy irradiation with X-rays. After treatment, the wells were reassembled and the cultures incubated at 37°C in a humidified atmosphere (95% air, 5% CO₂) for 30 min (for measurement of early

changes in metabolic activity) and 18 h (for measurement of late changes in metabolic activity).

After each incubation period, 10 μ l of 5 mg/ml MTT solution (prepared by dissolving MTT powder in PBS) was then added to each well and incubated at 37°C in a humidified atmosphere (95% air, 5% CO₂) for 4 h in the dark. In this period, metabolically active cells reduce MTT to generate purple formazan crystals. One hundred microlitres of dimethyl sulfoxide (DMSO) was then added to each well to solubilise the crystals, and the plate was incubated for 10 min at room temperature in the dark. The absorbances (optical densities, OD) were then determined for samples and blanks at a wavelength of 560 nm, using the microplate spectrophotometer (Labtech International, Sussex, UK; Model #: LT-4000).

Data were expressed as relative metabolic activities, given as the ratios of mean absorbances in samples treated with X-rays to those obtained for the negative controls (OD_{6Gy}/OD_{0Gy}); or the ratios of mean absorbances in triplicate samples treated with combinations of X-rays and RFF to those obtained for the positive controls ($OD_{6Gy+RFF}/OD_{0Gy+RFF}$). The ratios of the former to the latter (or modifying factors) were then taken to represent the mode by which RFF modified metabolic activity in irradiated cells. A modifying factor of >1, 1, or <1 indicates a reduction, no effect, or an enhancement in metabolic activity in irradiated cells by RFF, respectively.

2.10. Measurement of Cytosolic Superoxide Dismutase (SOD) Activity

To evaluate the role of superoxide radicals generated in RFF-mediated cellular response to X-ray treatment, cells were seeded in 25 cm² tissue culture flasks and incubated until they reached ~80% confluence. The cells were subsequently exposed to a 1000-Hz modulated radiofrequency field for 30 min, 2 h prior to or following a 6 Gy X-ray irradiation. The field was chosen for this assay as it showed the largest effect on radiosensitivity in all cell lines. The treated cells were incubated at 37°C in a humidified atmosphere (95% air, 5% CO₂) for 2 h. The cells were then harvested by gentle trypsinisation. One million cells were harvested and washed 3 times by centrifugation in ice cold PBS at 250 × *g* for 10 min and discarding the supernatant. The cells were then resuspended in 0.5 ml of ice cold PBS and homogenised, using a BeadBug 3 microtube homogeniser (Whitehead Scientific, Western Cape, South Africa; Serial #: 1184070298). Homogenisation was performed in plastic tubes containing 1.5 mm high impact zirconium beads at 4000 RPM in 3 bursts each 30 seconds long with a 30 second rest between bursts. The cells were then centrifuged at 1500 × *g* for 10 minutes at 4°C and the supernatants collected. The supernatants were spun again at 10 000 × *g* for 15 min at 4°C and the supernatants collected to measure cytosolic SOD 1,Cu/Zn.

Standards were prepared by serial dilution from the stock provided with the SOD kit (Life Technologies Corporation, Frederick, MD, USA; cat # EIASODC) to achieve concentrations of 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 U/ml. The samples were diluted at a ratio of 1:1 (sample:dilution buffer, v/v). Ten microlitres each of diluted samples and standards were pipetted, in triplicate, into pre-labelled wells of a 96-well plate, 50 µl of

1X substrate was added to each well, followed by 25 μ l of 1X xanthine oxidase, and the plate was incubated at 25°C for 20 min. The plates were then read at 450 nm on a spectrophotometer (Labtech International, Sussex, UK; Model #: LT-4000). A standard curve was plotted from the absorbances obtained from the standard samples, and was used to determine sample concentrations of SOD.

Data were further expressed as relative SOD activities, given as the ratios of mean SOD concentrations in triplicate samples treated with X-rays to those obtained for the negative controls (SOD_{6Gy}/SOD_{0Gy}); or the ratios of mean concentrations in samples treated with combinations of X-rays and RFF to those obtained for the positive controls ($SOD_{6Gy+1000Hz}/SOD_{1000Hz}$ or $SOD_{1000Hz+6Gy}/SOD_{1000Hz}$). The ratios of the former to the latter (or modifying factors) were then taken to represent the mode by which RFF modified SOD activity in irradiated cells. A modifying factor of >1, 1, or <1 indicates a reduction, no effect, or an enhancement in SOD activity in irradiated cells by RFF, respectively.

2.11. Assessment of DNA Damage Response

To evaluate the role of RFF exposure in radiation-induced DNA damage response, the micronucleus formation assay was used (Akudugu et al., 2000). For this, exponentially growing cells were trypsinised into single-cell suspensions and seeded (40 000 cells per plate) into 35-mm plastic petri dishes containing 22 mm glass cover slips, to a final medium volume of 2 ml, and incubated at 37°C in a humidified atmosphere (95% air, 5% CO₂) for 2 h. The cells were exposed to radiofrequency fields (100 or 1000 Hz) and X-rays (0 or 2 Gy) as in Sections 2.4 and 2.5. Immediately after exposure, and not

later than 30 min, the cultures were treated with cytochalasin-B dissolved in DMSO to a final concentration of 2 µg/ml and incubated.

In a preliminary micronucleus experiment, using the lung fibroblasts (L132), it was observed that a typical 40-h incubation yielded a large proportion of multi-nucleated cells (Akudugu et al., 2000). As the micronucleus assay requires that micronuclei be scored in only binucleated cells, it was necessary to determine the doubling times of all cell lines to guide the timing of subsequent experiments. The doubling times for the L132, MeWo, Be11, and DU145 cell lines were found to be 21, 47, 28, and 30 h, respectively. Therefore, the cell cultures were correspondingly terminated after being incubated for 24, 48, 42, and 42 h, respectively. For this, the samples were fixed with a methanol:acetic acid mixture (3:1, v/v), air-dried and stained with acridine orange, and the cover slips mounted on glass microscope slides for fluorescence microscopy. Micronuclei in binucleated cells were counted, using a fluorescence microscope (LABOPHOT-2, Nikon, Japan; serial #: 465393). At least 500 binucleated cells were evaluated per experiment. Micronucleus frequency (MNF) was expressed as the mean number of micronuclei per binucleated cell. The proportion of binucleated cells and the nucleation index, BNI (proportion of cells with 2 main nuclei) were determined as indicators of cell proliferation.

A micronucleus frequency-based RFF treatment related modifying factor was derived as follows: $MF_{MNF} = (MNF_{2Gy} - MNF_{medium}) / (MNF_{2Gy+RFF} - MNF_{RFF})$ or $(MNF_{2Gy} - MNF_{medium}) / (MNF_{RFF+2Gy} - MNF_{RFF})$, where MNF_{medium} and MNF_{RFF} are the micronucleus frequencies of the growth medium and RFF controls, respectively. The corresponding modifying factor, based on binucleation, was given as: $MF_{BNI} =$

$(\text{BNI}_{2\text{Gy}}/\text{BNI}_{\text{medium}})/(\text{BNI}_{2\text{Gy}+\text{RFF}}/\text{BNI}_{\text{RFF}})$ or $(\text{BNI}_{2\text{Gy}}/\text{BNI}_{\text{medium}})/(\text{BNI}_{\text{RFF}+2\text{Gy}}/\text{BNI}_{\text{RFF}})$. A modifying factor of >1 , 1 , or <1 indicates a reduction, no effect, or an enhancement in MNF or BNI in irradiated cells by RFF, respectively.

2.12. Data analysis

Data analyses were performed with GraphPad Prism software (San Diego, USA). All data were presented as the mean (\pm SEM) from three independent experiments or triplicate samples. Where applicable, errors were determined using appropriate error propagation formulae. The unpaired two-sided *t*-test was used to compare two data sets. A $P < 0.05$ indicates a statistically significant difference between the data sets. For associations, linear regression analyses were used.

CHAPTER 3

3. Results

3.1. Effect of Radiofrequency Field Exposure on Plating Efficiency

To determine if radiofrequency field exposure alone affects colony forming capacity, the plating efficiencies (PE) of the cell lines in negative (medium only) and positive (RFF exposed) controls were compared to obtain a modifying factor (MF_{PE}) as follows: $MF_{PE} = PE_{\text{medium}}/PE_{\text{RFF}}$. The MF_{PE} -values for the various frequencies are presented in Table 3.1.

Table 3.1: Summary of plating efficiency modifying factors for DU145, MeWo, Be11, and L132 following exposure to 100, 1000, 2000, and 4000-Hz modulated radiofrequency fields. Data are presented as the mean \pm SEM from 3 independent experiments.

Cell line	100 Hz	1000 Hz	2000 Hz	4000 Hz
DU145	1.00 \pm 0.03	0.99 \pm 0.02	0.97 \pm 0.04	1.02 \pm 0.06
MeWo	1.08 \pm 0.15	0.94 \pm 0.03	0.91 \pm 0.02	0.91 \pm 0.06
Be11	1.03 \pm 0.05	1.12 \pm 0.06	0.94 \pm 0.06	0.88 \pm 0.04
L132	0.94 \pm 0.03	0.98 \pm 0.04	0.94 \pm 0.08	0.95 \pm 0.05

Except for Be11, which seemed to exhibit an enhanced plating efficiency when exposed to the 4000-Hz modulated field, there was no apparent effect on the plating efficiency of all cell lines for all frequencies. With modifying factors very close to 1.0, it can be inferred that RFF treatment alone at these frequencies is not cytotoxic.

3.2. Radiosensitivity and Radiomodulatory Effect of Radiofrequency Fields

To determine cellular intrinsic radiosensitivity and evaluate the effect of radiofrequency fields on radiosensitivity, the clonogenic cell survival assay was used. The data for each cell line and frequency are described below:

DU145

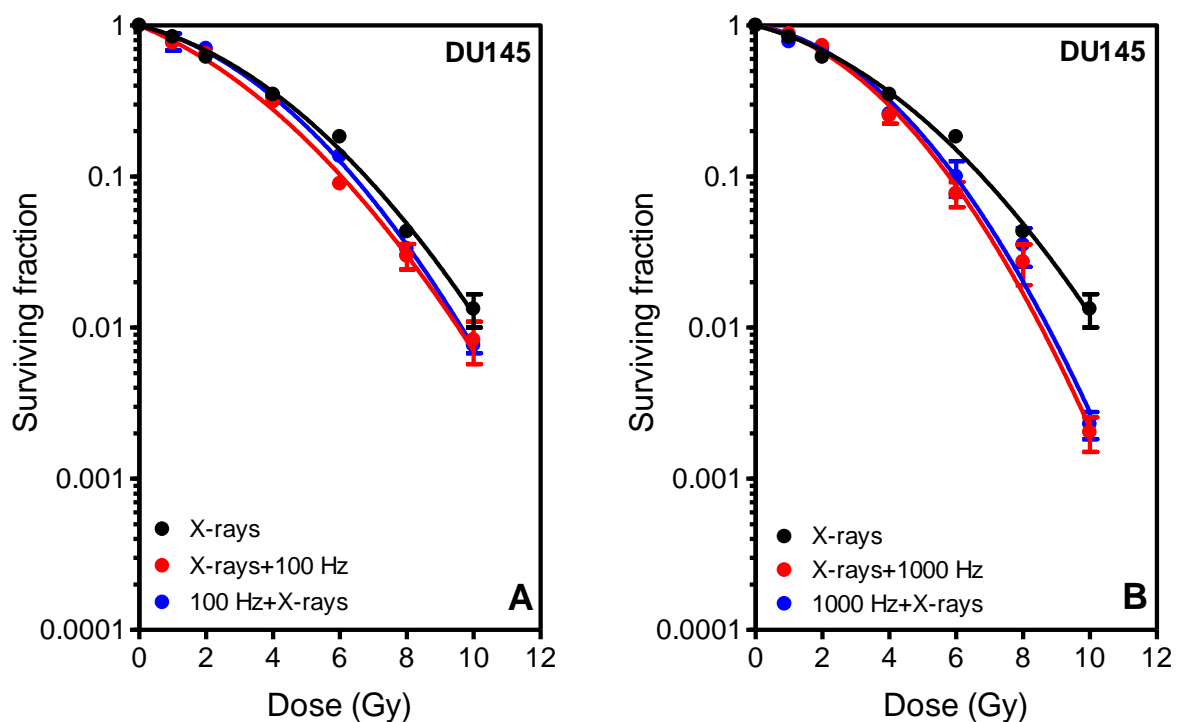


Figure 3.1: Clonogenic survival curves for the DU145 cell line after X-ray irradiation alone (black) and in combination with 100-Hz (A) and 1000-Hz (B) modulated radiofrequency fields (RFF). RFF exposure was performed 2 h prior to (blue) or after (red) X-ray treatment. The survival curves were obtained by fitting data from three independent experiments to the linear-quadratic model (Equation 2.2).

The dose response curves for the prostate cancer cell line are presented in Figure 3.1. The radiobiological parameters are summarised in Table 3.2. The data show that the 100-Hz modulated field either had no effect or slightly enhanced radiosensitivity at doses between 2 and 6 Gy (Figure 3.1A, Table 3.2). Treatment of cells with this

radiofrequency field prior to and after 2 Gy of X-rays resulted in 1% ($P = 0.8307$) and 9% ($P = 0.0621$) more cell killing, respectively. Similarly, the corresponding reductions in cell survival at 6 Gy were 2% ($P = 0.0579$) and 4% ($P = 0.0099$). The mean inactivation doses following RFF exposure before and after X-ray treatment also did not differ markedly from that for X-ray exposure alone. These data suggest that exposure of the DU145 cells to the 100-Hz modulated RFF after X-ray irradiation was more radiosensitising than when cells were pre-exposed to this radiofrequency field, but the differences were not statistically significant ($0.0507 \leq P \leq 0.1050$). The slight sensitisation, or lack thereof, is also reflected by the modifying factor not differing significantly from 1.0, as presented in Table 3.3.

Table 3.2: Summary of radiobiological parameters for the DU145 cell line. SF_2 and SF_6 denote the surviving fraction at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. \bar{D} denotes the mean inactivation dose (area under the cell survival curve). Data are presented as the mean \pm SEM from 3 independent experiments.

Treatment	SF_2	SF_6	\bar{D} (Gy)	α (Gy^{-1})	β (Gy^{-2})
X-rays	0.68 \pm 0.03	0.15 \pm 0.01	3.44 \pm 0.07	0.13 \pm 0.03	0.03 \pm 0.00*
100 Hz + X-rays	0.67 \pm 0.03	0.13 \pm 0.01	3.36 \pm 0.11	0.13 \pm 0.02	0.04 \pm 0.00*
X-rays + 100 Hz	0.59 \pm 0.01	0.11 \pm 0.01	3.13 \pm 0.02	0.20 \pm 0.03	0.03 \pm 0.01
1000 Hz + X-rays	0.69 \pm 0.10	0.10 \pm 0.02	3.16 \pm 0.14	0.08 \pm 0.06	0.06 \pm 0.01
X-rays + 1000 Hz	0.67 \pm 0.09	0.09 \pm 0.02	3.23 \pm 0.12	0.10 \pm 0.06	0.05 \pm 0.01

*errors less than 0.01; actual values were used for error propagation in Table 3.3.

Table 3.3: Modifying factors (MF), relative to X-ray treatment alone, derived from the SF_2 , SF_6 , and \bar{D} values presented in Table 3.2 for the DU145 cell line according to Equation (2.4). Errors were calculated using error propagation formulae for ratios.

Treatment	MF_{SF_2}	MF_{SF_6}	$MF_{\bar{D}}$
100 Hz + X-rays	1.01 ± 0.06	1.15 ± 0.12	1.02 ± 0.04
X-rays + 100 Hz	1.15 ± 0.05	1.36 ± 0.15	1.10 ± 0.02
1000 Hz + X-rays	0.99 ± 0.15	1.50 ± 0.32	1.09 ± 0.05
X-rays + 1000 Hz	1.02 ± 0.14	1.67 ± 0.39	1.07 ± 0.05

When cells were treated with the 1000-Hz field in combination with X-rays, radiosensitisation was more pronounced at doses higher than 2 Gy, as shown by the much more prominent “bendiness” of combination curves compared to the X-ray only curve (Figure 3.1B). At 2 Gy both pre- and post-exposure to this field did not influence the radiosensitivity, yielding modifying factors of ~ 1.0 (Table 3.3). On the other hand, treatment of cells with RFF prior to and after 6 Gy of X-rays resulted in 5% ($P = 0.0684$) and 6% ($P = 0.0212$) more cell killing, respectively (Table 3.2), with corresponding modifying factors of greater than 1.0 (Table 3.3). Regardless of the parameter considered, there was no significant difference in radiosensitisation between pre- and post-exposure to this RFF ($0.6221 \leq P \leq 0.8543$).

The data in Table 3.2 show that the X-rays+100 Hz treatment yield a ~ 1.5 -fold larger linear component of cell killing ($\alpha = 0.20 \text{ Gy}^{-1}$) than those for the 100 Hz+X-rays and X-ray treatments ($\alpha = 0.13 \text{ Gy}^{-1}$). The β -components of cell killing for these treatments are similar. The α/β ratios for the 100 Hz+X-rays and X-rays+100 Hz were found to be

3.25 ± 0.54 and 6.67 ± 2.44 , respectively, and did not differ from that for the X-ray treatment alone (4.33 ± 1.11 Gy). However, the 1000 Hz+X-rays and X-rays+1000 Hz treatments resulted in decreased linear and increased quadratic components of cell killing. The resulting α/β ratios were 1.33 ± 1.02 and 2.00 ± 1.26 Gy, respectively, indicating a ~2-fold reduction in ratio in the 1000-Hz modulated RFF treatment.

MeWo

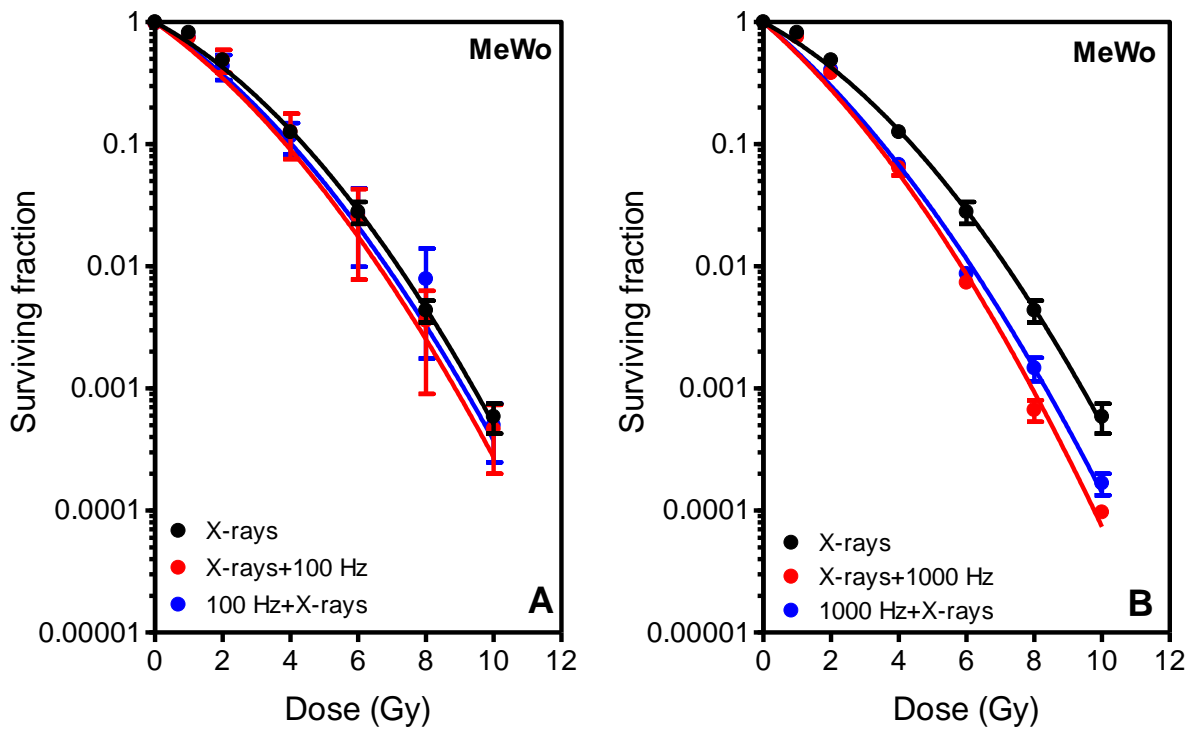


Figure 3.2: Clonogenic survival curves for the MeWo cell line after X-ray irradiation alone (black) and in combination with 100-Hz (A) and 1000-Hz (B) modulated radiofrequency fields (RFF). RFF exposure was performed 2 h prior to (blue) or after (red) X-ray treatment. The survival curves were obtained by fitting data from three independent experiments to the linear-quadratic model (Equation 2.2).

The cell survival curves in Figure 3.2 show that pre- and post-exposure to the 100-Hz modulated radiofrequency wave had no effect on radiosensitivity in the MeWo cell line, regardless of radiation dose (Figure 3.2A). Most all of the derived parameters, namely,

SF_2 , SF_6 , \bar{D} , α , and β were found to be similar for both treatment sequences (Table 3.4), giving modifying factors of approximately 1.0 (Table 3.5).

Table 3.4: Summary of radiobiological parameters for the MeWo cell line. SF_2 and SF_6 denote the surviving fraction at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. \bar{D} denotes the mean inactivation dose (area under the cell survival curve). Data are presented as the mean \pm SEM from 3 independent experiments.

Treatment	SF_2	SF_6	\bar{D} (Gy)	α (Gy ⁻¹)	β (Gy ⁻²)
X-rays	0.43 \pm 0.04	0.03 \pm 0.00*	3.37 \pm 0.03	0.34 \pm 0.04	0.04 \pm 0.01
100 Hz + X-rays	0.39 \pm 0.10	0.03 \pm 0.02	2.20 \pm 0.31	0.42 \pm 0.12	0.04 \pm 0.01
X-rays + 100 Hz	0.37 \pm 0.10	0.03 \pm 0.02	2.28 \pm 0.32	0.46 \pm 0.12	0.04 \pm 0.02
1000 Hz + X-rays	0.30 \pm 0.03	0.01 \pm 0.00*	2.10 \pm 0.07	0.53 \pm 0.05	0.04 \pm 0.01
X-rays + 1000 Hz	0.28 \pm 0.02	0.01 \pm 0.00*	2.01 \pm 0.04	0.56 \pm 0.05	0.04 \pm 0.01

*errors less than 0.01; actual values were used for error propagation in Table 3.5.

Pre- and post-exposure to the 1000-Hz modulated RFF led to lower cell survival at low and high doses (Figure 3.2B). Treatment of the MeWo cells with this RFF prior to and after 2 Gy of X-rays led to 13% ($P = 0.0657$) and 15% ($P = 0.0277$) more cell killing, respectively (Table 3.4). This translates to radiosensitivity modifying factors of greater than 1.0 (Table 3.5). Correspondingly, either treatment combination at 6 Gy resulted in a 2% ($0.0040 \leq P \leq 0.0078$) more cell killing, yielding dose modifying factors of greater than 2.0. The mean inactivation doses for RFF exposure before and after X-ray treatment were found to be significantly lower than that for X-ray treatment alone ($0.0025 \leq P \leq 0.0191$). However, based on all parameters, there was no statistically

significant difference between RFF exposure before and after X-ray treatment ($0.1269 \leq P \leq 0.5750$).

Table 3.5: Modifying factors (MF), relative to X-ray treatment alone, derived from the SF_2 , SF_6 , and \bar{D} values presented in Table 3.4 for the MeWo cell line according to Equation (2.4). Errors were calculated using error propagation formulae for ratios.

Treatment	MF_{SF_2}	MF_{SF_6}	$MF_{\bar{D}}$
100 Hz + X-rays	1.10 ± 0.30	1.00 ± 0.56	1.53 ± 0.22
X-rays + 100 Hz	1.16 ± 0.33	1.16 ± 0.71	1.48 ± 0.21
1000 Hz + X-rays	1.43 ± 0.20	2.42 ± 0.32	1.61 ± 0.06
X-rays + 1000 Hz	1.54 ± 0.18	3.22 ± 0.49	1.68 ± 0.04

The absence of a modulatory effect in the 100 Hz+X-rays and X-rays+100 Hz treatment can also be due to their respective α/β ratios (10.50 ± 3.99 and 11.50 ± 6.48 Gy) not differing markedly from that of the X-ray treatment ($\alpha/\beta = 8.50 \pm 2.35$ Gy). The α coefficients for the pre-exposure and post-exposure of MeWo cells to the 1000-Hz modulated RFF were about 1.5-fold larger than that of the X-ray only treatment (Table 3.4). The corresponding α/β ratios were 13.25 ± 3.54 and 14.00 ± 3.72 Gy.

Be11

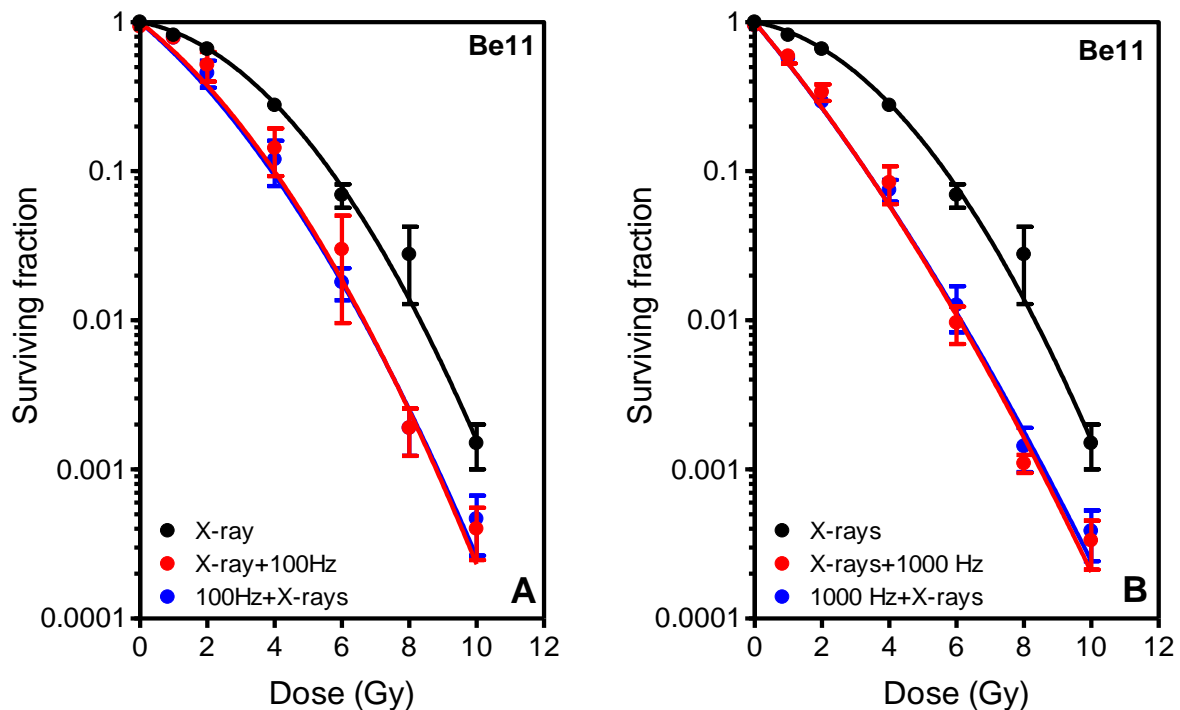


Figure 3.3: Clonogenic survival curves for the Be11 cell line after X-ray irradiation alone (black) and in combination with 100-Hz (A) and 1000-Hz (B) modulated radiofrequency fields (RFF). RFF exposure was performed 2 h prior to (blue) or after (red) X-ray treatment. The survival curves were obtained by fitting data from three independent experiments to the linear-quadratic model (Equation 2.2).

The survival curves in Figure 3.3 show that pre- and post-exposure of Be11 cells to RFF modulated by 100 and 1000 Hz are rendered more radiosensitive compared to when the cells were exposed to X-rays only. This is evident by the much steeper (~ 4.4 - to 6.9 -fold larger α -component of cell killing) survival curves in combination treatments compared to the survival curves from X-ray only (Table 3.6). The β -coefficients in all cases varied over a narrow range (0.02 - 0.06 Gy $^{-2}$). The resulting α/β ratios for the 100 Hz+X-rays, X-rays+100 Hz, 1000 Hz+X-rays, and X-rays+1000 Hz treatments were 11.00 ± 3.55 , 10.00 ± 3.91 , 31.00 ± 16.01 , and 30.00 ± 15.65 Gy, respectively, while that for the X-ray only treatment emerged as 1.50 ± 1.19 Gy.

Table 3.6: Summary of radiobiological parameters for the Be11 cell line. SF_2 and SF_6 denote the surviving fraction at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. \bar{D} denotes the mean inactivation dose (area under the cell survival curve). Data are presented as the mean \pm SEM from 3 independent experiments.

Treatment	SF_2	SF_6	\bar{D} (Gy)	α (Gy^{-1})	β (Gy^{-2})
X-rays	0.67 \pm 0.05	0.08 \pm 0.01	3.06 \pm 0.03	0.09 \pm 0.07	0.06 \pm 0.01
100 Hz + X-rays	0.36 \pm 0.04	0.02 \pm 0.01	2.24 \pm 0.28	0.44 \pm 0.09	0.04 \pm 0.01
X-rays + 100 Hz	0.41 \pm 0.11	0.03 \pm 0.01	2.38 \pm 0.34	0.40 \pm 0.12	0.04 \pm 0.01
1000 Hz + X-rays	0.26 \pm 0.04	0.01 \pm 0.00*	1.67 \pm 0.12	0.62 \pm 0.08	0.02 \pm 0.01
X-rays + 1000 Hz	0.27 \pm 0.03	0.01 \pm 0.00*	1.77 \pm 0.06	0.61 \pm 0.07	0.02 \pm 0.01

*errors less than 0.01; actual values were used for error propagation in Table 3.7.

The radiosensitivity modifying efficacy of the 100- and 1000-Hz modulated radiofrequency fields was not affected by sequences of treatment (i.e. whether RFF exposure occurred before or after X-ray treatment), as the respective cell survival curves were congruent (Figure 3.3). Based on SF_2 , SF_6 , and \bar{D} , the 1000-Hz modulated field was ~1.45-, ~1.76-, and ~1.33-fold more potent in radiosensitising the Be11 cells than the 100-Hz modulated field (Table 3.7).

Table 3.7: Modifying factors (MF), relative to X-ray treatment alone, derived from the SF_2 , SF_6 , and \bar{D} values presented in Table 3.6 for the Be11 cell line according to Equation (2.4). Errors were calculated using error propagation formulae for ratios.

Treatment	MF_{SF_2}	MF_{SF_6}	$MF_{\bar{D}}$
100 Hz + X-rays	1.86 ± 0.25	4.10 ± 1.42	1.37 ± 0.17
X-rays + 100 Hz	1.63 ± 0.46	3.28 ± 1.55	1.29 ± 0.18
1000 Hz + X-rays	2.58 ± 0.44	6.31 ± 2.22	1.83 ± 0.13
X-rays + 1000 Hz	2.48 ± 0.33	6.83 ± 2.07	1.73 ± 0.06

L132

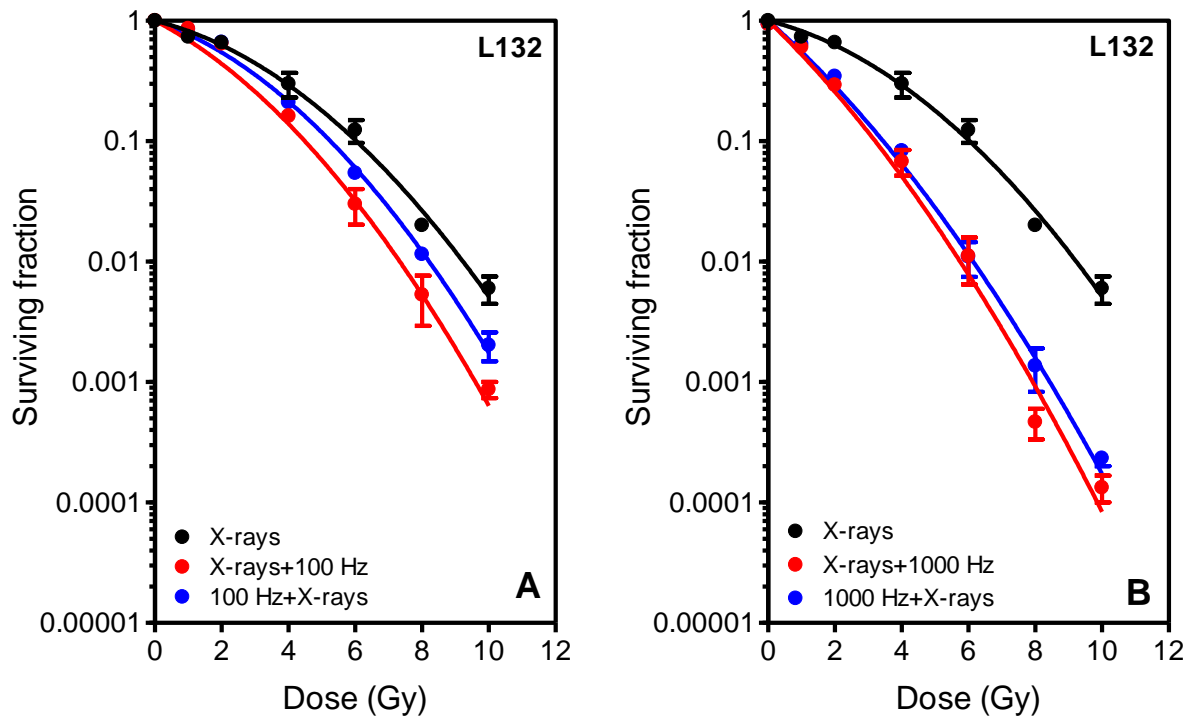


Figure 3.4: Clonogenic survival curves for the L132 cell line after X-ray irradiation alone (black) and in combination with 100-Hz (A) and 1000-Hz (B) modulated radiofrequency fields (RFF). RFF exposure was performed 2 h prior to (blue) or after (red) X-ray treatment. The survival curves were obtained by fitting data from three independent experiments to the linear-quadratic model (Equation 2.2).

From Figure 3.4, it is apparent that both 100-Hz and 1000-Hz modulated fields have radiomodulatory effects on L132 cells, as evidenced by the steeper cell survival curves from the combination treatments when compared to that of the X-ray only treatment. Table 3.8 summarises the radiobiological parameters for these treatments. Exposure to the 100-Hz modulated field prior to or after X-ray treatment led to 17% ($P = 0.0168$) and 32% ($P = 0.1221$) reduction in SF_2 , respectively (Figure 3.4A, Table 3.8). The corresponding decreases in SF_6 were 50% ($P = 0.0838$) and 67% ($P = 0.0364$). Combined treatment also reduced the mean inactivation dose by up to 18%, although this reduction did not reach statistical significance ($P \geq 0.2275$).

Table 3.8: Summary of radiobiological parameters for the L132 cell line. SF_2 and SF_6 denote the surviving fraction at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. \bar{D} denotes the mean inactivation dose (area under the cell survival curve). Data are presented as the mean \pm SEM from 3 independent experiments.

Treatment	SF_2	SF_6	\bar{D} (Gy)	α (Gy ⁻¹)	β (Gy ⁻²)
X-rays	0.66 \pm 0.07	0.12 \pm 0.02	3.33 \pm 0.42	0.16 \pm 0.05	0.04 \pm 0.01
100 Hz + X-rays	0.55 \pm 0.02	0.06 \pm 0.00*	2.90 \pm 0.08	0.22 \pm 0.03	0.04 \pm 0.00*
X-rays + 100 Hz	0.45 \pm 0.08	0.04 \pm 0.01	2.72 \pm 0.11	0.33 \pm 0.09	0.04 \pm 0.01
1000 Hz + X-rays	0.29 \pm 0.04	0.01 \pm 0.00*	1.82 \pm 0.07	0.56 \pm 0.07	0.03 \pm 0.01
X-rays + 1000 Hz	0.25 \pm 0.04	0.01 \pm 0.00*	1.67 \pm 0.04	0.61 \pm 0.08	0.03 \pm 0.01

*errors less than 0.01; actual values were used for error propagation in Table 3.9.

Exposure to the 1000-Hz modulated radiofrequency field, regardless of sequence, further sensitised L132 cells to X-rays (Figure 3.4B, Table 3.8). SF_2 , SF_6 , and \bar{D} were reduced by up to 62% ($0.0071 \leq P \leq 0.0086$), 92% ($0.0111 \leq P \leq 0.0127$), and 50% ($0.0170 \leq P \leq 0.0245$), respectively. The modifying factors obtained from these parameters are summarised in Table 3.9. Based on SF_2 , SF_6 , and \bar{D} , combined treatment with the 1000-Hz modulated RFF was found to be correspondingly 1.9-, 4.5, and 1.6-fold more potent, respectively, in radiosensitising the L132 cells than the 100-Hz modulated field.

Table 3.9: Modifying factors (MF), relative to X-ray treatment alone, derived from the SF_2 , SF_6 , and \bar{D} values presented in Table 3.8 for the L132 cell line according to Equation (2.4). Errors were calculated using error propagation formulae for ratios.

Treatment	MF_{SF_2}	MF_{SF_6}	$MF_{\bar{D}}$
100 Hz + X-rays	1.20 ± 0.13	1.90 ± 0.40	1.15 ± 0.15
X-rays + 100 Hz	1.47 ± 0.30	3.22 ± 1.12	1.22 ± 0.16
1000 Hz + X-rays	2.28 ± 0.40	9.67 ± 3.14	1.83 ± 0.24
X-rays + 1000 Hz	2.64 ± 0.51	12.89 ± 3.14	1.99 ± 0.26

The radiosensitisation seen in the 100 Hz+X-rays and X-rays+100 Hz treatments was also apparent in the increase in the α/β ratio from 4.00 ± 1.60 Gy (X-ray only) to 5.50 ± 0.92 and 8.25 ± 3.05 Gy, respectively. The more pronounced radiosensitisation in the 1000 Hz+X-rays and X-rays+1000 Hz treatments was reflected in even larger ratios of 18.67 ± 6.65 and 20.33 ± 7.28 , respectively.

3.3. Summary of Relative Cellular Radiosensitivity

To obtain an overall indication of the relative radiosensitivity of the cell lines, a rank order was constructed based on SF_2 , SF_6 , and \bar{D} , as presented in Table 3.10. Except for combined treatment with the 1000-Hz modulated radiofrequency field, \bar{D} emerged as the parameter providing the most consistent ranking of radiosensitivity. Using the frequency of cell lines under each rank for X-ray treatment alone, they may be arranged in order of increasing radiosensitivity as: DU145 \rightarrow L132 \rightarrow Be11 \rightarrow MeWo.

Table 3.10: Summary of relative radiosensitivity of DU145, L132, Be11, and MeWo cell lines based on SF_2 , SF_6 , and \bar{D} .

Treatment	Parameter	Increasing Radiosensitivity →			
X-rays	SF_2	DU145	Be11	L132	MeWo
	SF_6	DU145	L132	Be11	MeWo
	\bar{D}	DU145	L132	Be11	MeWo
100 Hz + X-rays	SF_2	DU145	L132	MeWo	Be11
	SF_6	DU145	L132	MeWo	Be11
	\bar{D}	DU145	L132	Be11	MeWo
X-rays + 100 Hz	SF_2	DU145	L132	Be11	MeWo
	SF_6	DU145	L132	Be11	MeWo
	\bar{D}	DU145	L132	Be11	MeWo
1000 Hz + X-rays	SF_2	DU145	MeWo	L132	Be11
	SF_6	DU145	MeWo	L132	Be11
	\bar{D}	DU145	MeWo	L132	Be11
X-rays + 1000 Hz	SF_2	DU145	MeWo	Be11	L132
	SF_6	DU145	Be11	MeWo	L132
	\bar{D}	DU145	MeWo	Be11	L132

Radiosensitivity ranking for the combined treatment with the 100-Hz modulated RFF was similar to the intrinsic radiosensitivity ranking. The ranking obtained from the combined treatment with the 1000-Hz modulated RFF markedly differed from the intrinsic ranking, and emerged as DU145 → MeWo → L132 ≈ Be11, with the MeWo cells showing an increased treatment resistance.

3.4. Effect of Radiofrequency Fields Modulated at Higher Frequencies on Radiosensitivity

To assess whether the radiosensitisation seen in concomitant treatment with radiofrequency fields modulated at 100 and 1000 Hz was persistent at fields modulated at higher frequencies, RFF modulated at 2000 and 4000 Hz were evaluated for their effect on cell survival at 2 and 6 Gy. The survival data for the DU145 cell line are presented in Figure 3.5.

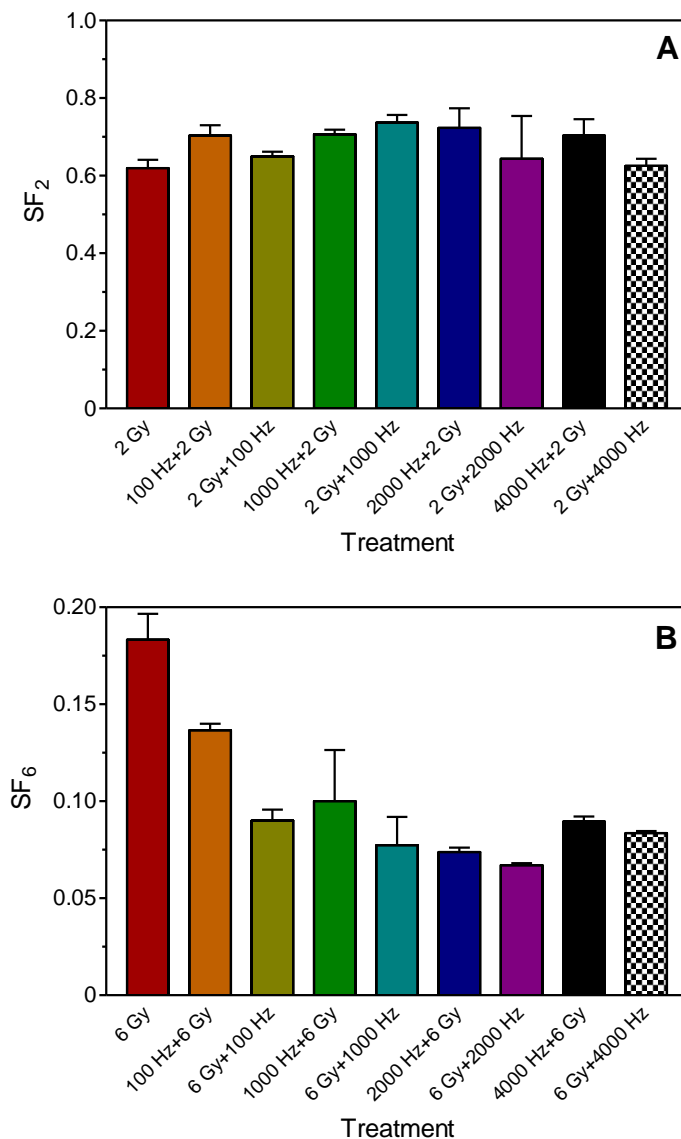


Figure 3.5: Cell survival data of the DU145 cell line for various radiofrequency field treatments at (A) 2 Gy and (B) 6 Gy.

Use of higher modulatory frequencies did not affect radiosensitivity at 2 Gy (Figure 3.5A). However, the field modulated with 2000 Hz further radiosensitised the DU145 cells at 6 Gy, while the 4000-Hz modulated field resulted in a recovery of cell survival to levels comparable to those obtained for the 1000-Hz modulated field (Figure 3.5B).

The survival data for the MeWo cell line are shown in Figure 3.6. The 2000-Hz modulated field appears to induce some recovery in cell survival at 2 Gy (Figure 3.6A).

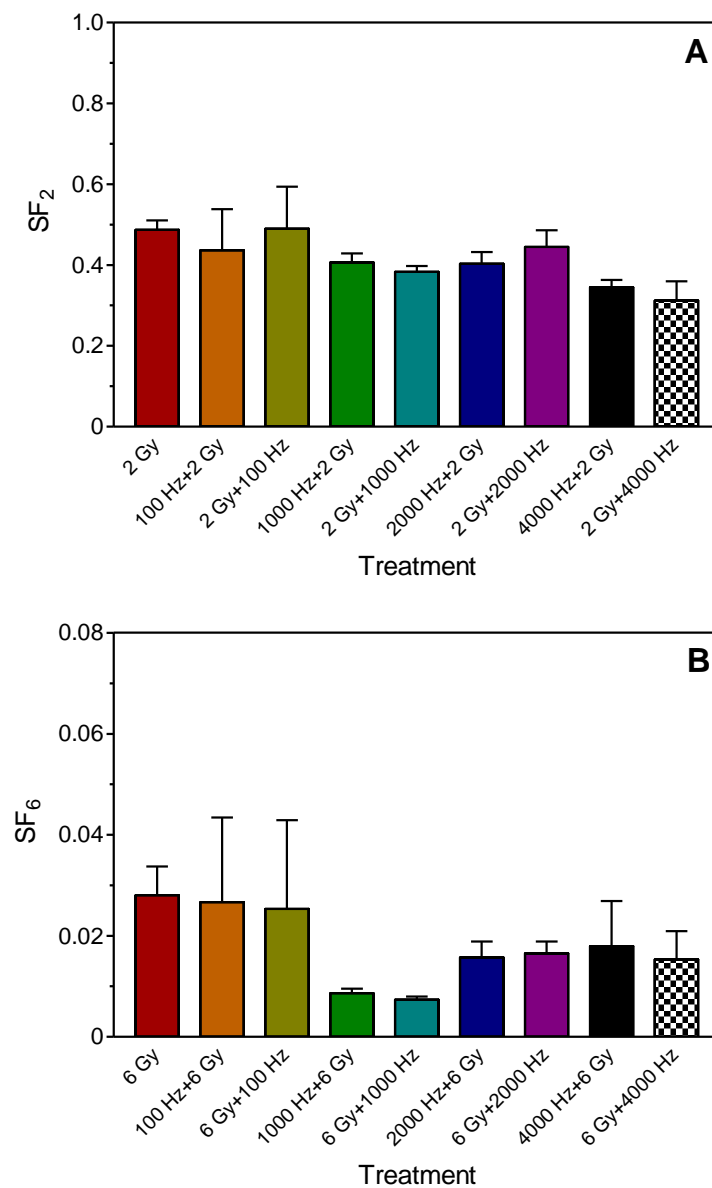


Figure 3.6: Cell survival data of the MeWo cell line for various radiofrequency field treatments at (A) 2 Gy and (B) 6 Gy.

On the other hand, use of a 4000-Hz modulated field led to an additional radiosensitisation relative to that obtained for the 1000-Hz modulated field. At 6 Gy, RFF modulated at 2000 and 4000 Hz induced a recovery in cell survival (Figure 3.6B).

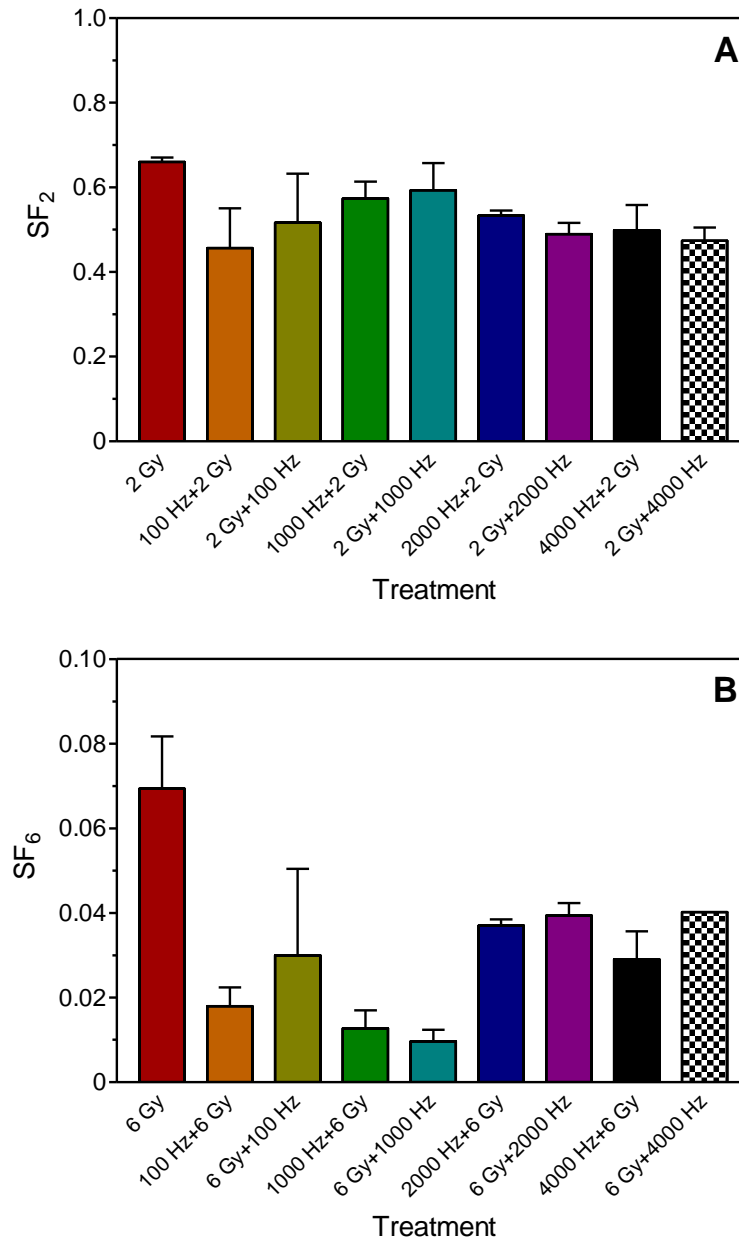


Figure 3.7: Cell survival data of the Be11 cell line for various radiofrequency field treatments at (A) 2 Gy and (B) 6 Gy.

Figure 3.7 shows the cell survival data for the Be11 cell line when treated with RFF modulated at various frequencies. At 2 Gy, fields modulated at 2000 and 4000 Hz

showed a slight radiosensitisation as seen for the 100-Hz modulated field (Figure 3.7A). Interestingly, combined treatment with higher frequency fields resulted in a pronounced recovery of cell survival at 6 Gy (Figure 3.7B).

From Figure 3.8, it is apparent that concomitant treatment of the L132 cell line with fields modulated at higher frequency (2000 and 4000 Hz) results in better cell survival at 2 and 6 Gy compared to 1000 Hz combination treatments.

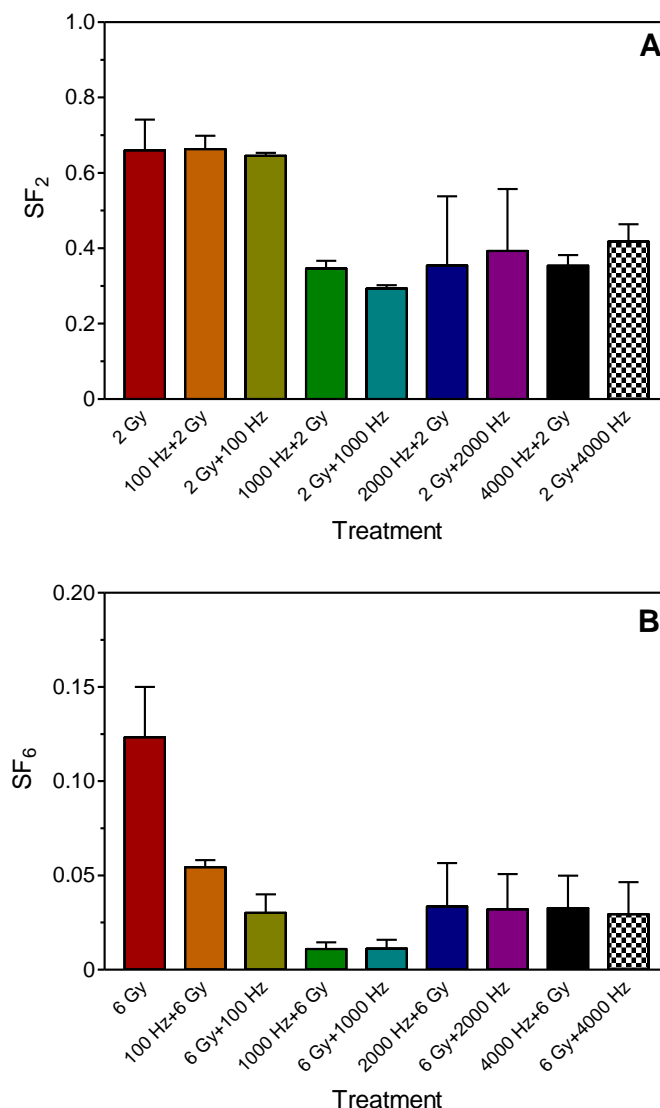


Figure 3.8: Cell survival data of the L132 cell line for various radiofrequency field treatments at (A) 2 Gy and (B) 6 Gy.

3.5. Effect of Radiofrequency Fields on Radiation-Induced Metabolic Changes

The MTT assay was used to assess the potential effect of the radiofrequency fields on early (30 min) and late (18 h) radiation-induced changes in metabolic activity of the four cell lines. It was further evaluated whether such changes play a role in long-term cell survival. For this, treatment-induced changes in metabolic activity was expressed as fold changes (relative metabolic activity) relative to appropriate controls (Section 2.9). These changes are presented as follows:

DU145

Figure 3.9 is an illustration of the fold changes in metabolic activity in the prostate cancer cell line, DU145, for the various treatments. While treatment with 6 Gy or 6 Gy+1000-Hz modulated RFF resulted in a reduction in early metabolic activity by about 60%, a 6 Gy+100-Hz modulated RFF treatment yielded an enhancement (~1.3-fold) in metabolic activity (Figure 3.9A). No effect on early metabolic activity was apparent, when cells were exposed to RFF prior to X-ray treatment.

Similarly, no effect on late metabolic activity was observed when cells were treated with either X-rays alone or X-rays followed by a 100-Hz modulated RFF (Figure 3.9B). However, exposure to a 100-Hz modulated field followed by X-ray treatment or an X-ray treatment followed by exposure to a 1000-Hz modulated RFF resulted in increased (~1.3-fold) late metabolic activity. In contrast to the absence of an effect at an early time point, pre-exposure to the 1000-Hz modulated field followed by X-ray treatment led to a reduction of ~32% in late metabolic activity (Figure 3.9B).

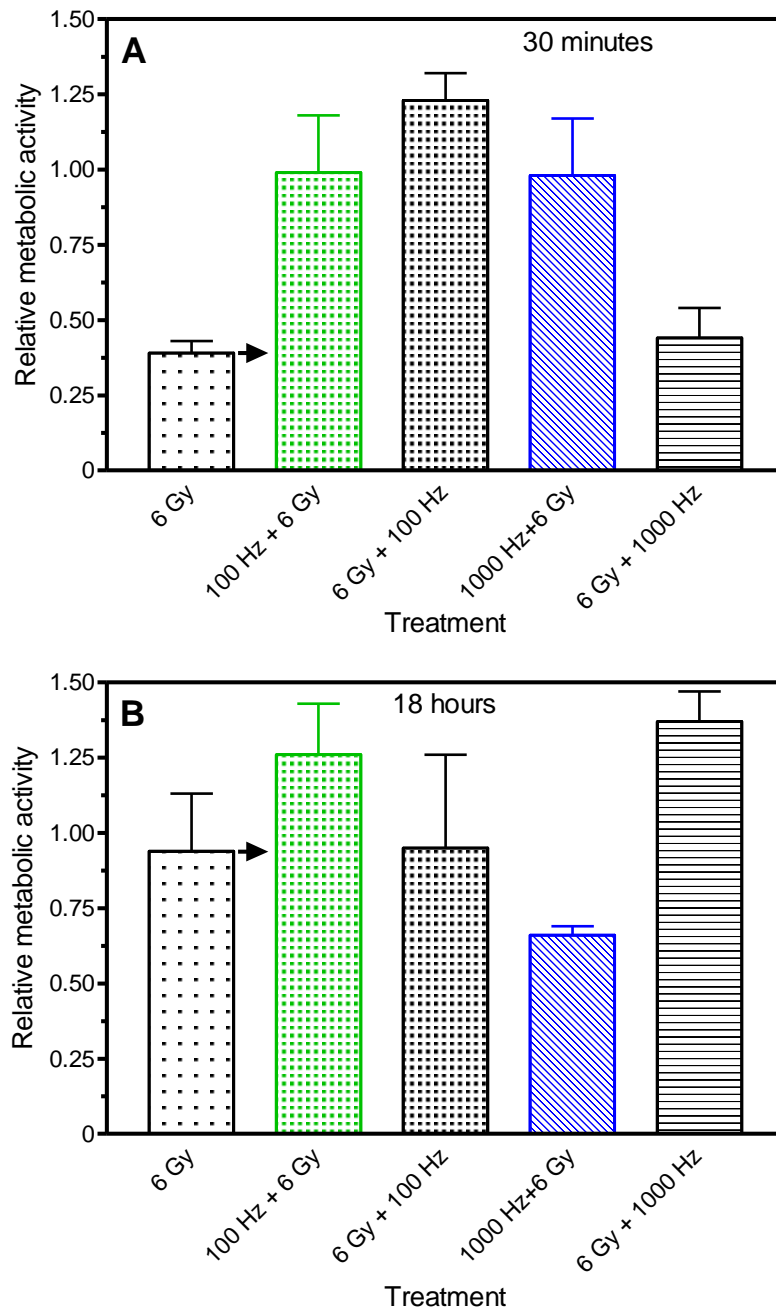


Figure 3.9: Relative metabolic activities for the prostate cancer cell line, DU145: (A) 30 min and (B) 18 h after treatment to 6 Gy of X-rays alone or in combination with 100-Hz and 1000-Hz modulated radiofrequency fields.

Determination of dose modifying factors (*MF*), as described in Section 2.9, revealed that regardless of RFF frequency and sequence of combination with X-rays, early metabolic activity was enhanced ($MF < 1.0$; Table 3.11). Late metabolic activity was

also enhanced in the 100-Hz modulated RFF+X-ray and X-ray+1000-Hz modulated RFF treatments, while a reduction ($MF > 1.0$; Table 3.11) was seen in the 1000-Hz modulated RFF+X-ray treatment. Exposure of cells to the 100-Hz modulated RFF after irradiation had no effect on late metabolic activity.

Table 3.11: Modifying factors (MF), relative to X-ray treatment alone, derived from the relative metabolic activities presented in Figure 3.9 for the DU145 cell line, as described in Section 2.9. Errors were calculated using error propagation formulae for ratios.

Treatment	30 min	18 h
100 Hz + 6 Gy	0.39 ± 0.09	0.75 ± 0.18
6 Gy + 100 Hz	0.32 ± 0.04	0.99 ± 0.38
1000 Hz + 6 Gy	0.40 ± 0.09	1.42 ± 0.29
6 Gy + 1000 Hz	0.89 ± 0.22	0.69 ± 0.15

MeWo

The data in Figure 3.10 show changes in metabolic activity in the melanoma cell line, MeWo, following the different treatments. Except for the X-ray+1000-Hz modulated RFF treatment that resulted in a reduction, the other treatments either had no effect or induced an increase in early metabolic activity (Figure 3.10A). Within experimental uncertainty, all treatments had no effect on late metabolic activity (Figure 3.10B).

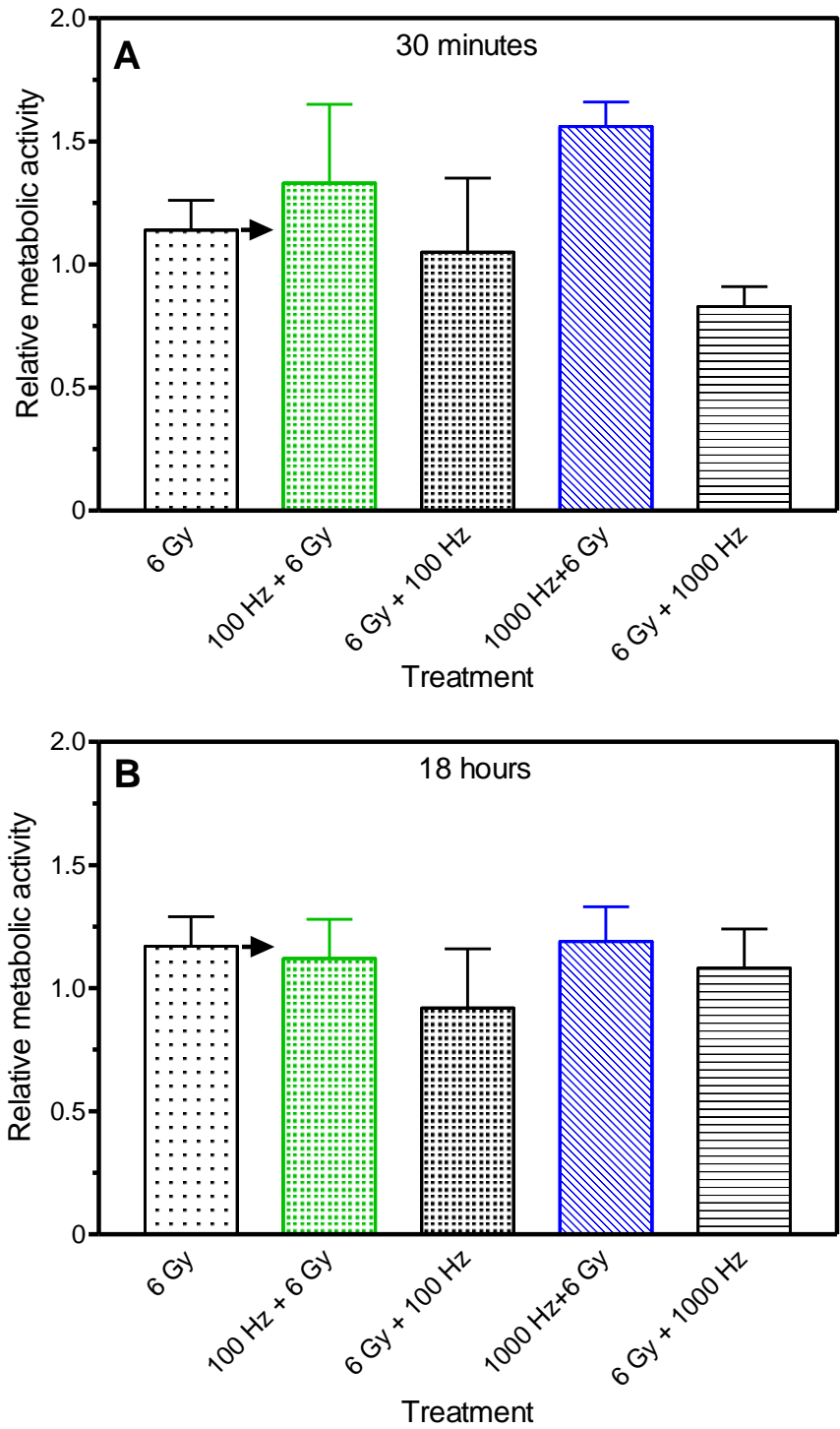


Figure 3.10: Relative metabolic activities for the melanoma cell line, MeWo: (A) 30 min and (B) 18 h after treatment to 6 Gy of X-rays alone or in combination with 100-Hz and 1000-Hz modulated radiofrequency fields.

The dose modifying factors presented in Table 3.12 demonstrate no marked changes in metabolic activity, except for the 100-Hz and 1000-Hz modulated RFF+X-ray treatments which resulted in ~1.2- and 1.4-fold enhancements in early metabolic activity, respectively, when compared with that of X-ray only treatment.

Table 3.12: Modifying factors (*MF*), relative to X-ray treatment alone, derived from the relative metabolic activities presented in Figure 3.10 for the MeWo cell line, as described in Section 2.9. Errors were calculated using error propagation formulae for ratios.

Treatment	30 min	18 h
100 Hz + 6 Gy	0.85 ± 0.22	1.05 ± 0.18
6 Gy + 100 Hz	1.09 ± 0.33	1.27 ± 0.36
1000 Hz + 6 Gy	0.73 ± 0.09	0.98 ± 0.15
6 Gy + 1000 Hz	1.37 ± 0.20	1.08 ± 0.20

Be11

X-ray treatment alone resulted in about 50% reduction and similar extent of enhancement in early and late metabolic activity in the Be11 cell line (Figure 3.11). Combined X-ray and RFF treatments of these cells, regardless of sequence, enhanced early metabolic activity (Figure 3.11A, Table 3.13). On the other hand, all combination treatments with both RFF led to a reduction in late metabolic activity (Figure 3.11B, Table 3.13).

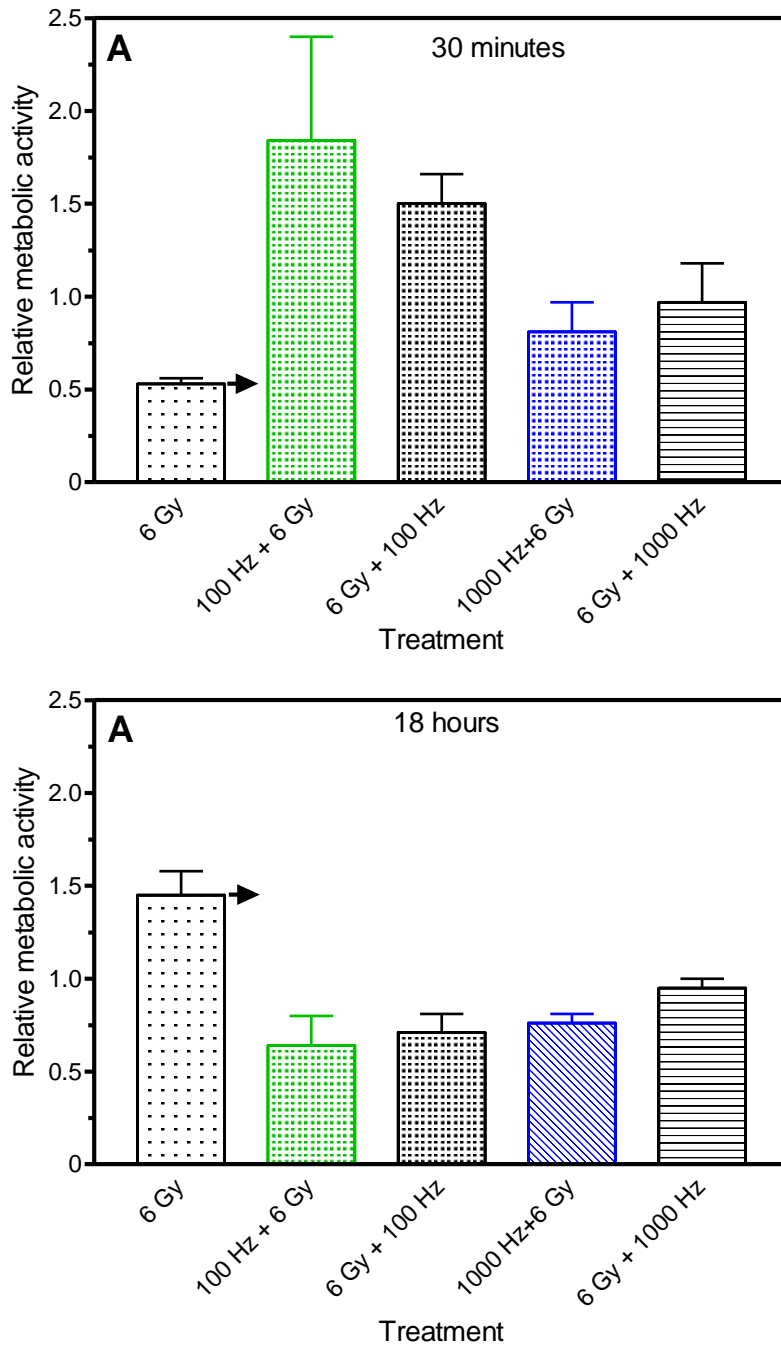


Figure 3.11: Relative metabolic activities for the melanoma cell line, Be11: (A) 30 min and (B) 18 h after treatment to 6 Gy of X-rays alone or in combination with 100-Hz and 1000-Hz modulated radiofrequency fields.

Table 3.13: Modifying factors (*MF*), relative to X-ray treatment alone, derived from the relative metabolic activities presented in Figure 3.11 for the Be11 cell line, as described in Section 2.9. Errors were calculated using error propagation formulae for ratios.

Treatment	30 min	18 h
100 Hz + 6 Gy	0.28 ± 0.09	2.27 ± 0.60
6 Gy + 100 Hz	0.35 ± 0.04	2.04 ± 0.34
1000 Hz + 6 Gy	0.65 ± 0.13	1.91 ± 0.21
6 Gy + 1000 Hz	0.55 ± 0.12	1.56 ± 0.16

L132

The relative metabolic activity data presented in Figure 3.12 show that X-ray irradiation of the normal lung fibroblasts, L132, resulted in about 60% increase in early metabolic activity (Figure 3.12A). Inclusion of RFF exposure either had no effect (6 Gy+100-Hz modulated RFF) or reduced (all other combined treatments) metabolic activity. On the other hand, a 6-Gy exposure alone resulted in about 40% reduction in late metabolic activity (Figure 3.12B). The 100-Hz modulated RFF+X-ray treatment enhanced late metabolic activity. However, the other combined treatments resulted in reduced activity, with the 1000-Hz modulated RFF being the most effective (Figure 3.12B).

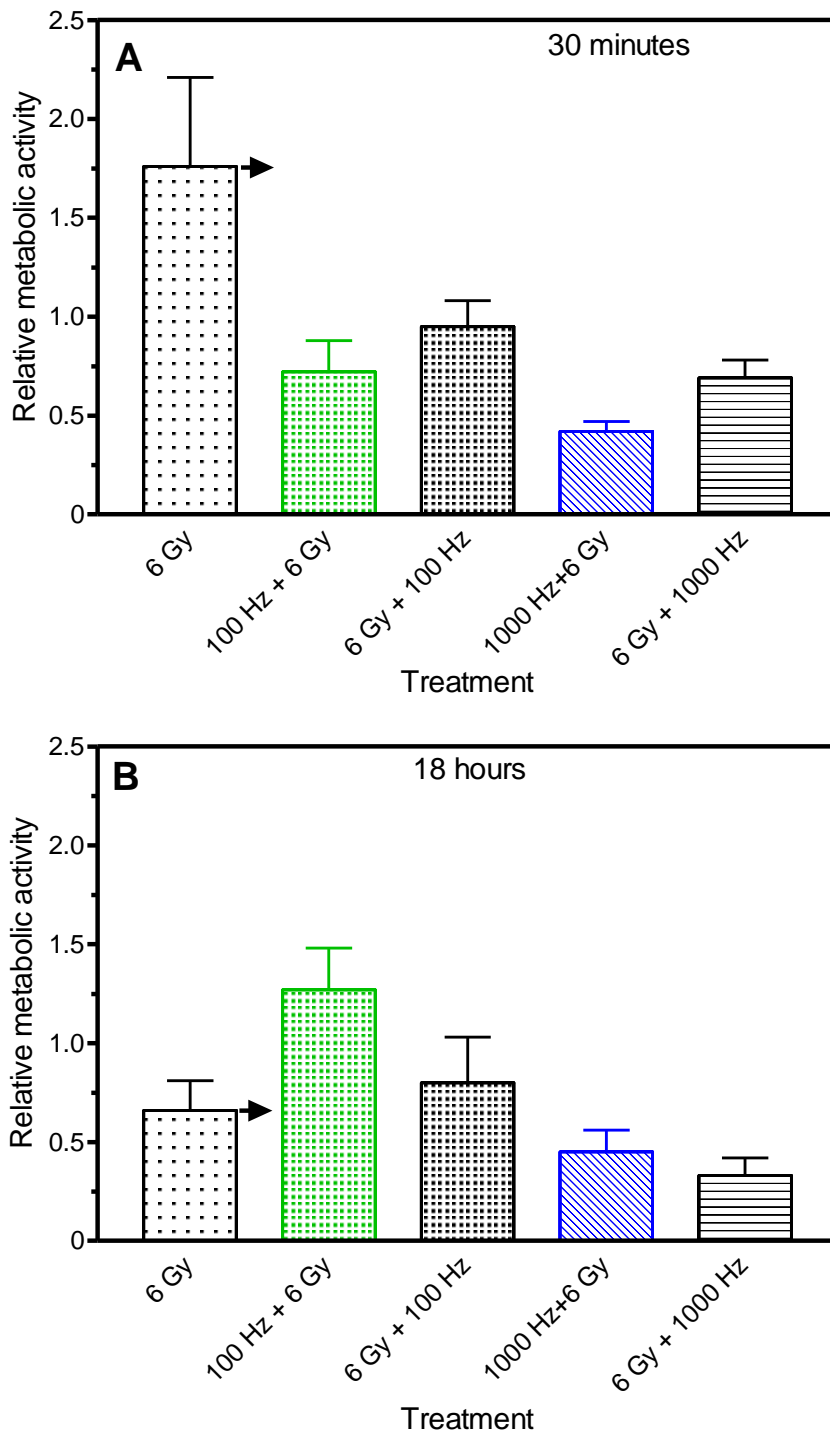


Figure 3.12: Relative metabolic activities for human lung fibroblasts, L132: (A) 30 min and (B) 18 h after treatment to 6 Gy of X-rays alone or in combination with 100-Hz and 1000-Hz modulated radiofrequency fields.

The dose modifying factors in Table 3.14 show that combined X-ray and RFF treatment of the lung fibroblasts, regardless of sequence, led to a reduction ($MF > 1.0$)

in early metabolic activity. A similar reduction in late metabolic activity was also seen when cells were concomitantly treated with the 1000-Hz modulated RFF. However, combined treatment with 100-Hz modulated RFF increased ($MF < 1.0$) late metabolic activity.

Table 3.14: Modifying factors (MF), relative to X-ray treatment alone, derived from the relative metabolic activities presented in Figure 3.12 for the L132 cell line, as described in Section 2.9. Errors were calculated using error propagation formulae for ratios.

Treatment	30 min	18 h
100 Hz + 6 Gy	2.44 ± 0.82	0.52 ± 0.15
6 Gy + 100 Hz	1.85 ± 0.54	0.83 ± 0.30
1000 Hz + 6 Gy	4.19 ± 1.18	1.47 ± 0.49
6 Gy + 1000 Hz	2.55 ± 0.75	2.00 ± 0.71

3.6. Relationship between Radiosensitivity and Metabolic Activity

To evaluate if treatment induced changes in metabolic activity play a role in the modulatory effects that radiofrequency fields have on cellular radiosensitivity, the modifying factors derived from the MTT assay were plotted as a function of those obtained from the clonogenic cell survival assay. The data in Figure 3.13 show a weak correlation [$Y=(0.14\pm 0.06)X+(0.20\pm 0.39)$; $R^2 = 0.4767$; $P = 0.058$] between the 100-Hz RFF induced radiosensitisation and changes in early metabolic activity (Figure 3.13A), where cell lines displaying high levels of radiosensitisation on the basis of

clonogenic survival tended to have reduced ($MF > 1.0$) metabolic activity. No relationship was apparent between early metabolic activity and cell survival when the 1000-Hz modulated RFF was employed.

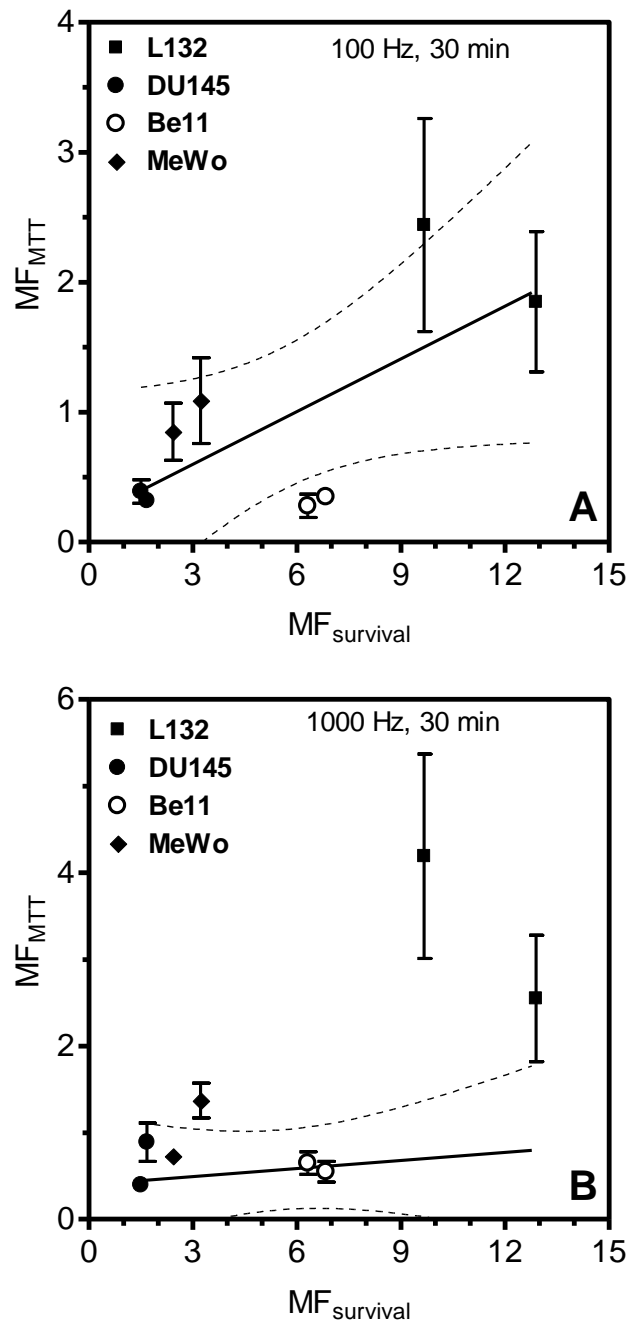


Figure 3.13: Plot of modifying factors from metabolic activity (measured 30 min after treatment) as a function of modifying factors from clonogenic cell survival for 4 cell lines: (A) combined treatment with 100-Hz modulated RFF and (B) combined treatment with 1000-Hz modulated RFF. Dashed lines represent the 95% confidence interval.

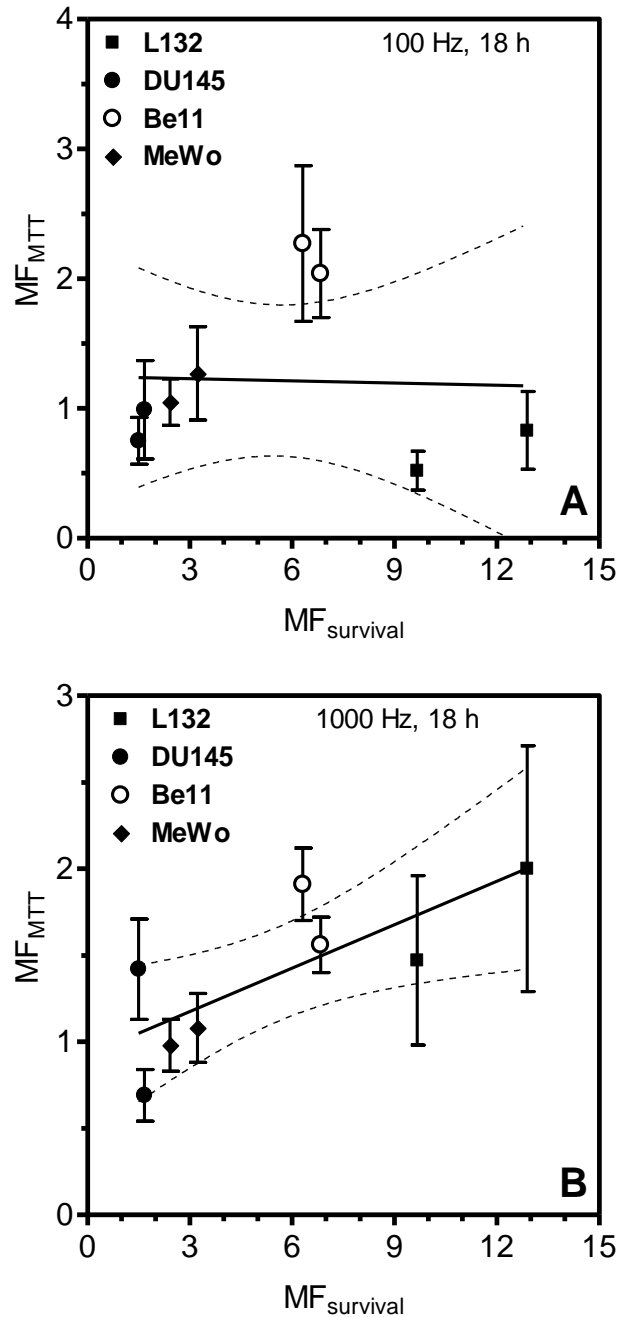


Figure 3.14: Plot of modifying factors from metabolic activity (measured 18 h after treatment) as a function of modifying factors from clonogenic cell survival for 4 cell lines: (A) combined treatment with 100-Hz modulated RFF and (B) combined treatment with 1000-Hz modulated RFF. Dashed lines represent the 95% confidence interval.

No link emerged between cell survival and late changes in metabolic activity for combined treatment with the 100-Hz modulated RFF (Figure 3.14A). However, if only the malignant cell lines are considered, a strong correlation is apparent, where an

increase in radiosensitisation is mirrored by a reduction in metabolic activity. Similarly, a correlation [$Y=(0.08\pm 0.03)X+(0.92\pm 0.20)$; $R^2 = 0.5851$; $P = 0.027$] emerged between changes in late metabolic activity and radiosensitisation by the 1000-Hz modulated radiofrequency field (Figure 3.14B).

3.7. Effect of a Radiofrequency Field on Radiation-Induced Changes in Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD) activity was measured in cell cultures, 2 h after treatment with 6 Gy of X-rays alone or in combination with a 1000-Hz modulated radiofrequency field. Figure 3.15 is the generated standard curve, as described under Section 2.10, from which SOD concentrations (an indicator of SOD activity) were determined.

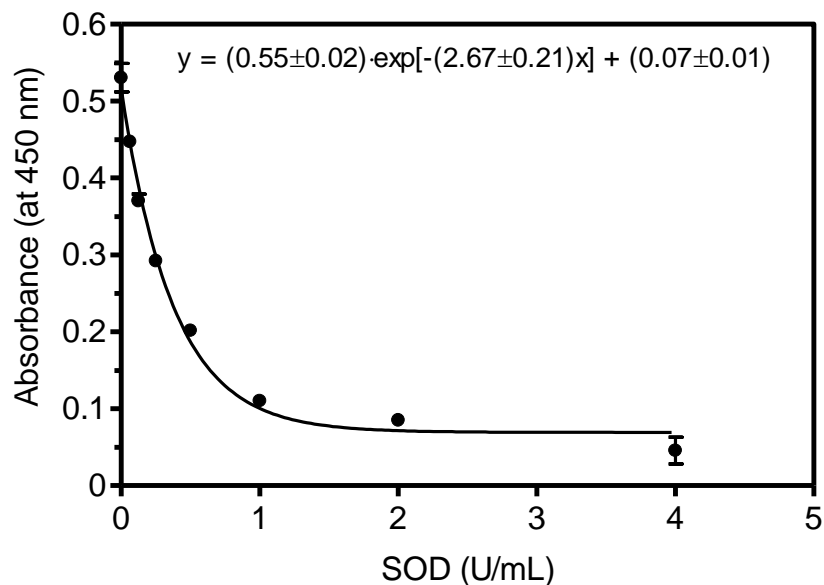


Figure 3.15: Standard curve used to determine the concentration of cytosolic superoxide dismutase (SOD) from absorbance measurements (Section 2.10).

The data in Figure 3.16 show that X-ray exposure alone resulted in ~40% increase in SOD activity in DU145 cells. Exposure of cells to the 1000-Hz modulated RFF led to ~2.7-fold increase in SOD activity, relative to the medium control. Relative to RFF exposure alone, X-ray irradiation prior to RFF treatment led to about 40% reduction in SOD activity, while a reversed treatment sequence increased SOD activity by a similar extent.

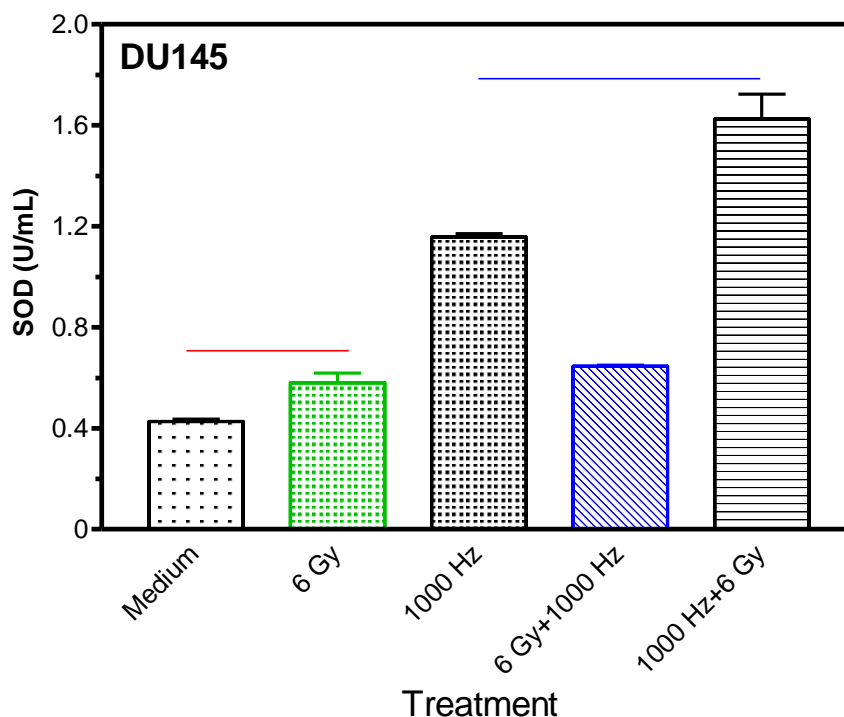


Figure 3.16: Superoxide dismutase concentration in DU145 cells following RFF exposure and X-ray irradiation singly or in combination. X-ray treatment was compared with negative control (medium; red horizontal line) and X-ray+RFF treatment was compared with positive control (1000 Hz; blue horizontal line), to generate relative SOD activities which were used to derive modifying factors, as described in Section 2.10.

Irradiation of the MeWo cells to 6 Gy yielded a 25% reduction in SOD activity, relative to the medium control (Figure 3.17). Relative to the medium control, RFF treatment alone resulted in only ~8% increase in SOD activity. The 6 Gy+1000-Hz modulated

RFF and 1000-Hz modulated RFF+6 Gy treatments also reduced SOD activity by ~25% and 16% relative to the 1000-Hz control, respectively.

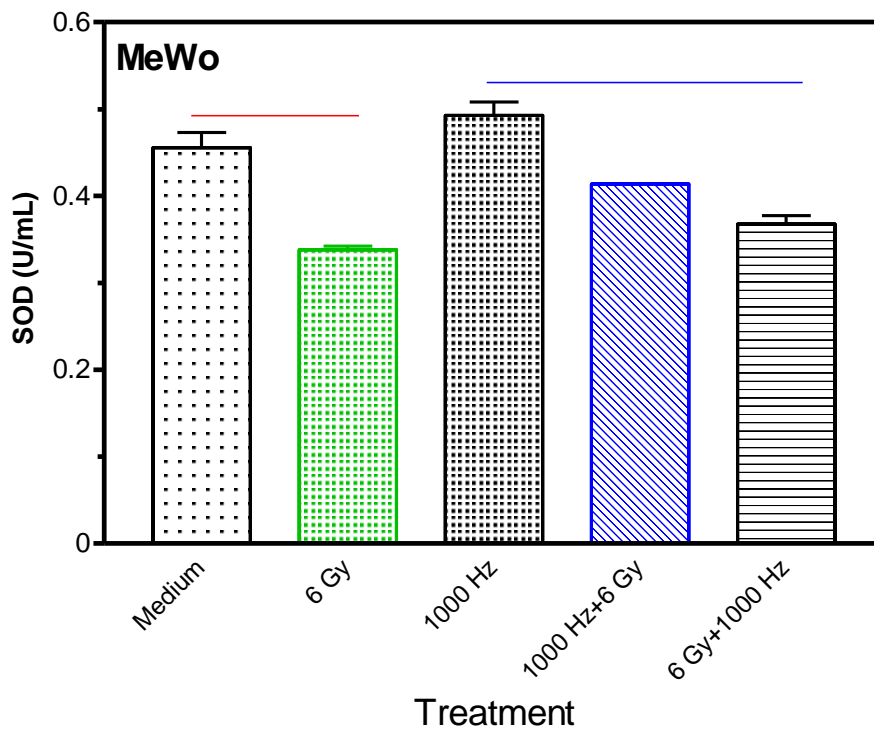


Figure 3.17: Superoxide dismutase concentration in MeWo cells following RFF exposure and X-ray irradiation singly or in combination. X-ray treatment was compared with negative control (medium; red horizontal line) and X-ray+RFF treatment was compared with positive control (1000 Hz; blue horizontal line), to generate relative SOD activities which were used to derive modifying factors, as described in Section 2.10.

The data shown in Figure 3.18 are the SOD activities in Be11 cells for the various treatments. Treatment of cells with 6 Gy or a 1000-Hz modulated RFF led to ~47% reduction or ~36% increase in SOD activity relative to the medium control, respectively. When compared to the 1000-Hz control, treatment with 6 Gy+1000-Hz modulated RFF and 1000-Hz modulated RFF+6 Gy reduced SOD activity by ~85% and 50%, respectively.

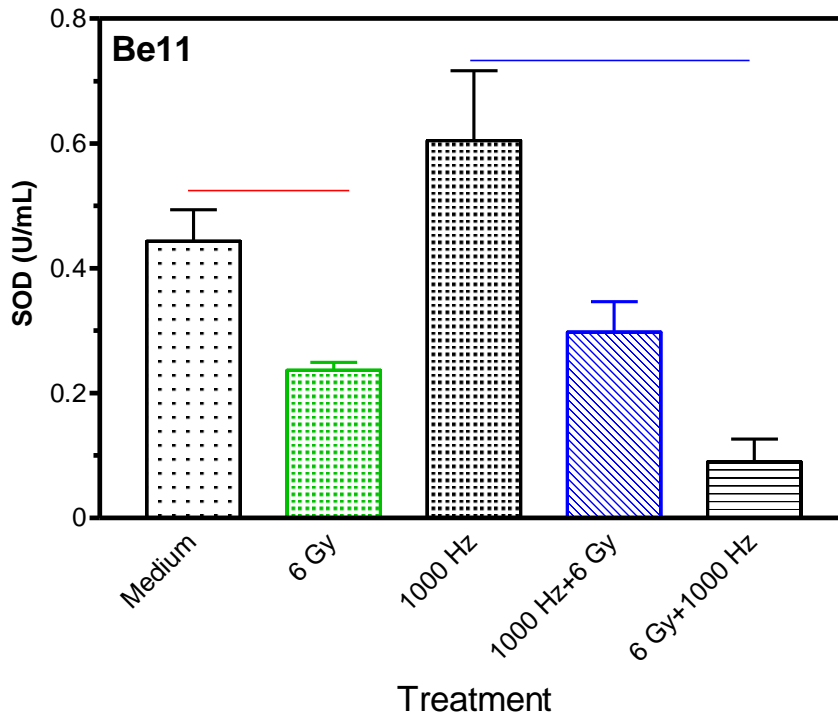


Figure 3.18: Superoxide dismutase concentration in Be11 cells following RFF exposure and X-ray irradiation singly or in combination. X-ray treatment was compared with negative control (medium; red horizontal line) and X-ray+RFF treatment was compared with positive control (1000 Hz; blue horizontal line), to generate relative SOD activities which were used to derive modifying factors, as described in Section 2.10.

When the normal lung fibroblasts were exposed to 6 Gy or the 1000-Hz modulated RFF, a slight increase of ~6% SOD activity was observed (Figure 3.19). Relative to the 1000-Hz control, 6 Gy+1000-Hz modulated RFF and 1000-Hz modulated RFF+6 Gy treatments resulted in ~8% and 11% reduction in SOD activity, respectively.

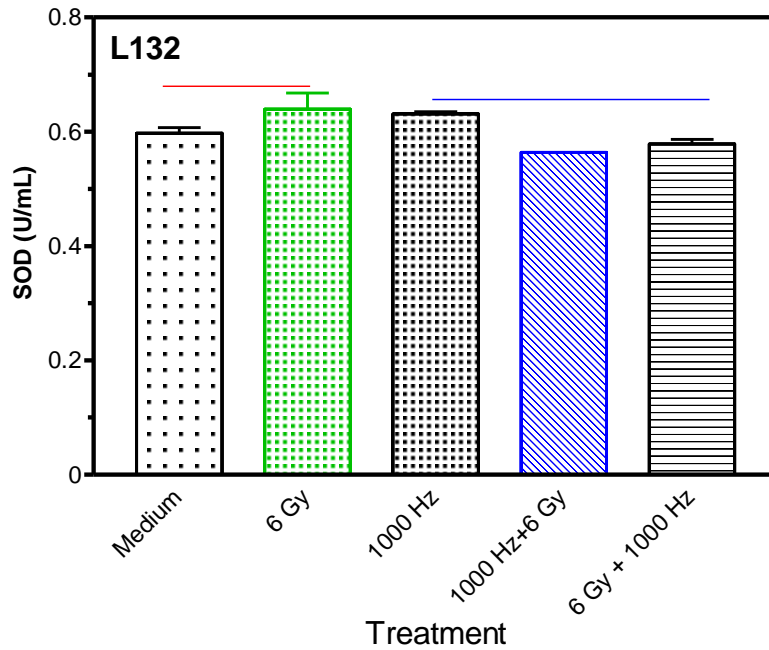


Figure 3.19: Superoxide dismutase concentration in L132 cells following RFF exposure and X-ray irradiation singly or in combination. X-ray treatment was compared with negative control (medium; red horizontal line) and X-ray+RFF treatment was compared with positive control (1000 Hz; blue horizontal line), to generate relative SOD activities which were used to derive modifying factors, as described in Section 2.10.

The dose modifying factors, on the basis of SOD activity, as described in Section 2.10, are presented in Table 3.15. For the DU145 cell line, treatment with a combination of the 1000-Hz modulated RFF and 6 Gy did not change SOD activity ($MF \approx 1.0$) when compared with the 6-Gy irradiation alone. However, when these cells were irradiated prior to RFF exposure, a larger than 2-fold reduction in SOD activity was observed. A similar treatment response was noted in the Be11 line.

For the MeWo cell line, pre-exposure to the RFF resulted in about 12% increase ($MF < 1.0$) in SOD activity. A treatment sequence reversal did not affect radiation-induced SOD activity.

For the lung fibroblasts, L132, both combination treatments yielded about 20% reduction ($MF > 1.0$) in SOD activity.

Table 3.15: Modifying factors (MF), relative to X-ray treatment alone, derived from the superoxide dismutase (SOD) activities presented in Figures 3.16-3.19 for the DU145, MeWo, Be11, and L132 cell lines, as described in Section 2.10. Errors were calculated using error propagation formulae for ratios.

Treatment	DU145	MeWo	Be11	L132
1000 Hz+X-rays	0.97 ± 0.09	0.88 ± 0.05	1.08 ± 0.30	1.20 ± 0.06
X-rays+1000 Hz	2.43 ± 0.18	0.99 ± 0.06	3.53 ± 1.71	1.16 ± 0.06

3.8. Relationship between Radiosensitivity and SOD Activity

To test whether treatment induced changes in superoxide dismutase (SOD) activity are linked to the modulatory effects seen in cellular radiosensitivity when the 1000-Hz modulated radiofrequency field (RFF) was applied, the modifying factors derived from SOD activity were plotted against those obtained from clonogenic cell survival. Figure 3.20 shows no relationship [$Y=(0.0018\pm 0.0935)X+(1.52\pm 0.63)$; $R^2 < 0.0001$; $P = 0.9856$] between the RFF induced radiosensitisation and changes in SOD activity.

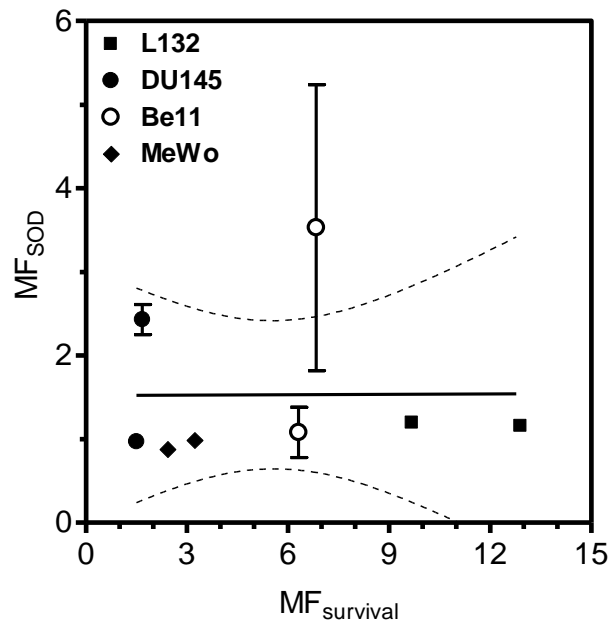


Figure 3.20: Plot of modifying factors from superoxide dismutase activity (measured 2 hours after treatment) as a function of modifying factors from clonogenic cell survival for 4 cell lines, following combined treatment with a 1000-Hz modulated RFF. Dashed lines represent the 95% confidence interval.

3.9. Effect of Radiofrequency Fields on Radiation Induced Micronucleus Frequency

The cytokinesis-block micronucleus assay was used to determine if radiofrequency fields had an effect on radiation induced DNA damage and block of cell proliferation. For this, treatment induced changes in micronucleus yield and binucleation were assessed. Data for the four cell lines are presented as follows:

DU145

The data presented in Figure 3.21 show that exposure of the prostate cancer cell line, DU145, to the 100-Hz and 1000-Hz modulated radiofrequency fields does not result in a significant change in background (medium control) micronucleus yield. Exposure of cells to the 100-Hz modulated RFF prior to or after 2 Gy of X-rays did not affect micronucleus yield (Figure 3.21A). While pre-exposure of cells to the 1000-Hz modulated RFF did not affect radiation induced micronucleus frequency, RFF exposure after irradiation resulted in ~18% reduction in DNA damage (Figure 3.21B).

The binucleation indices in the DU145 cells for the various treatments are presented in Figure 3.22. For the 100-Hz modulated RFF, no marked changes were observed in cellular capacity to go through a cell division (Figure 3.22A). Also, a 2-Gy of X-rays or a 1000-Hz modulated RFF treatment did not affect binucleation (Figure 3.22B). However, when this RFF field was combined with 2 Gy of X-rays, a reduction in cell proliferation of more than 30% was found.

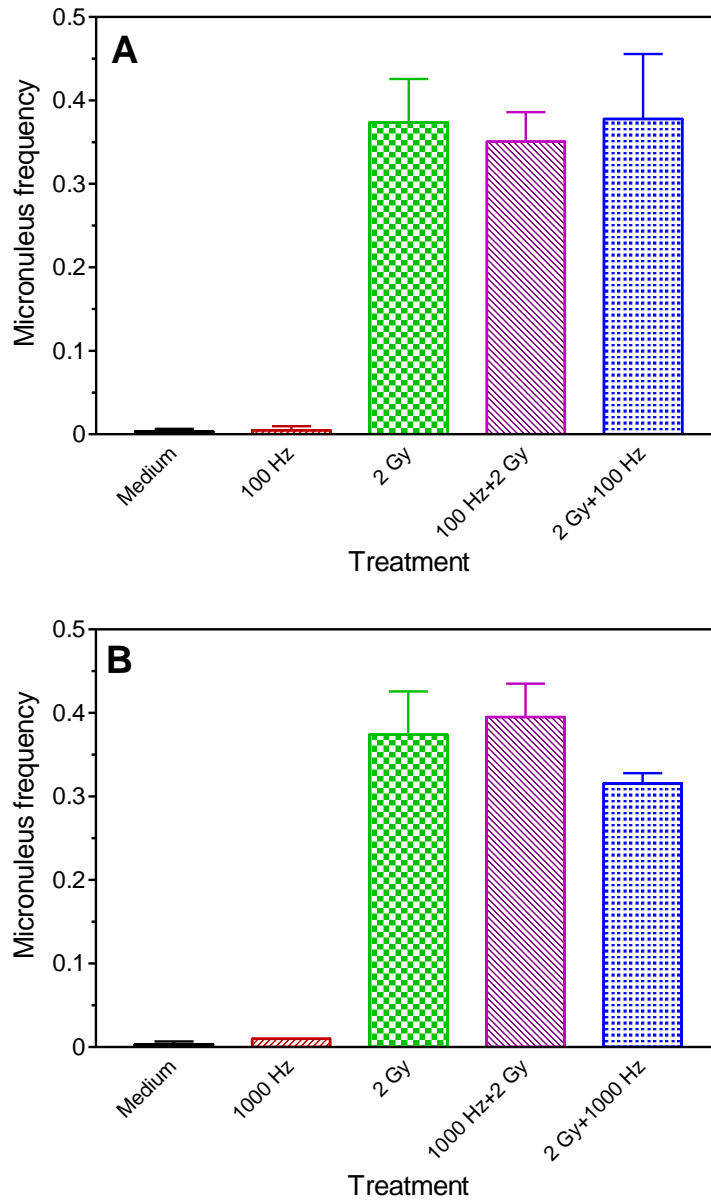


Figure 3.21: Micronucleus yield in the prostate cancer cell line, DU145, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field.

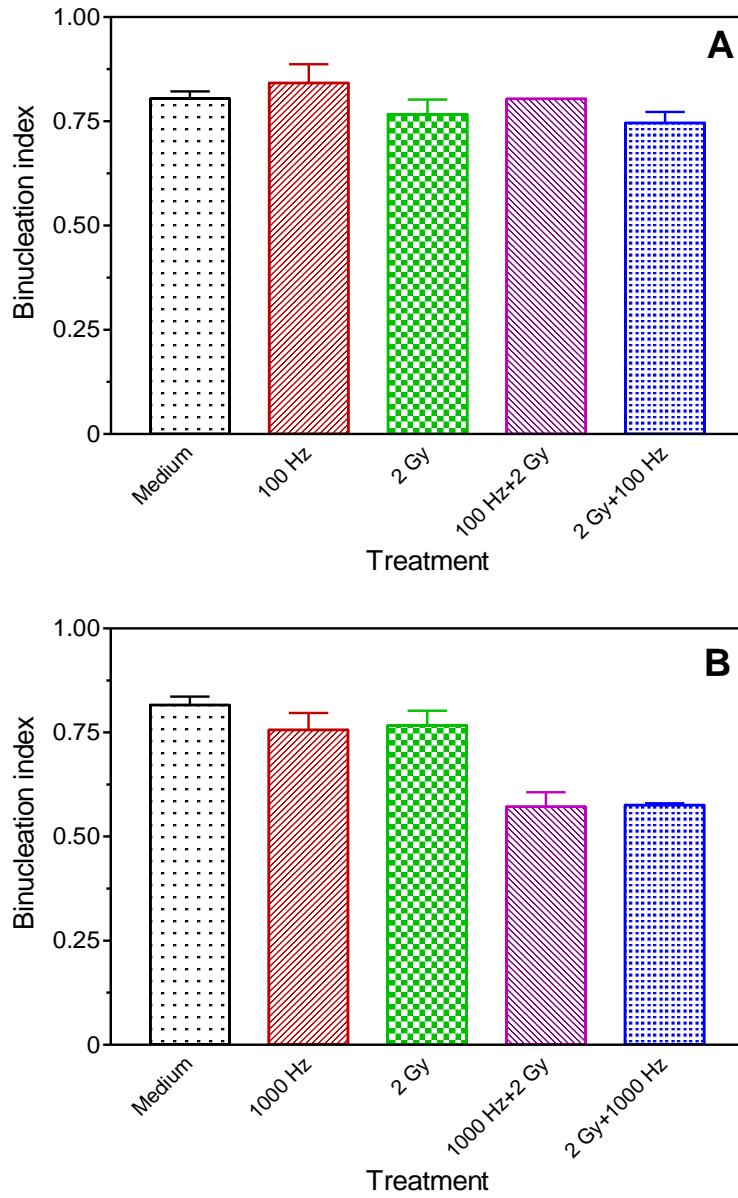


Figure 3.22: Binucleation index in the prostate cancer cell line, DU145, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field.

MeWo

Exposure of MeWo cells to both 100-Hz and 1000-Hz modulated RFF resulted in ~1.8-fold increase in micronucleus yield over background (Figure 3.23). Combined treatment with 2 Gy of X-rays and the 100-Hz modulated RFF led to ~1.2-fold increase in micronucleus yield, regardless of treatment sequence (Figure 3.23A).

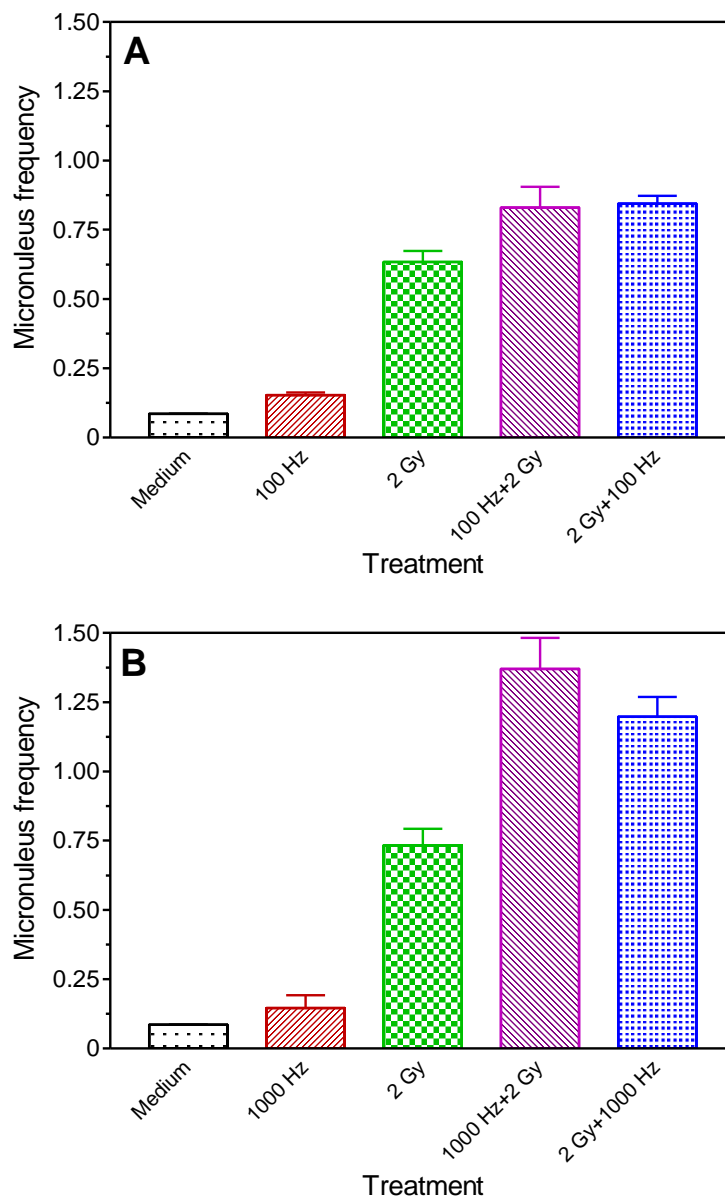


Figure 3.23: Micronucleus yield in the melanoma cell line, MeWo, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field.

When the 1000-Hz modulated RFF was combined with 2 Gy of X-rays, a 2-fold increase in DNA damage emerged (Figure 3.23B).

Figure 3.24A shows no effect on binucleation index when MeWo cells were exposed to X-rays or the 100-Hz modulated RFF alone. An approximately 18% reduction in binucleation was obtained from the combination treatments.

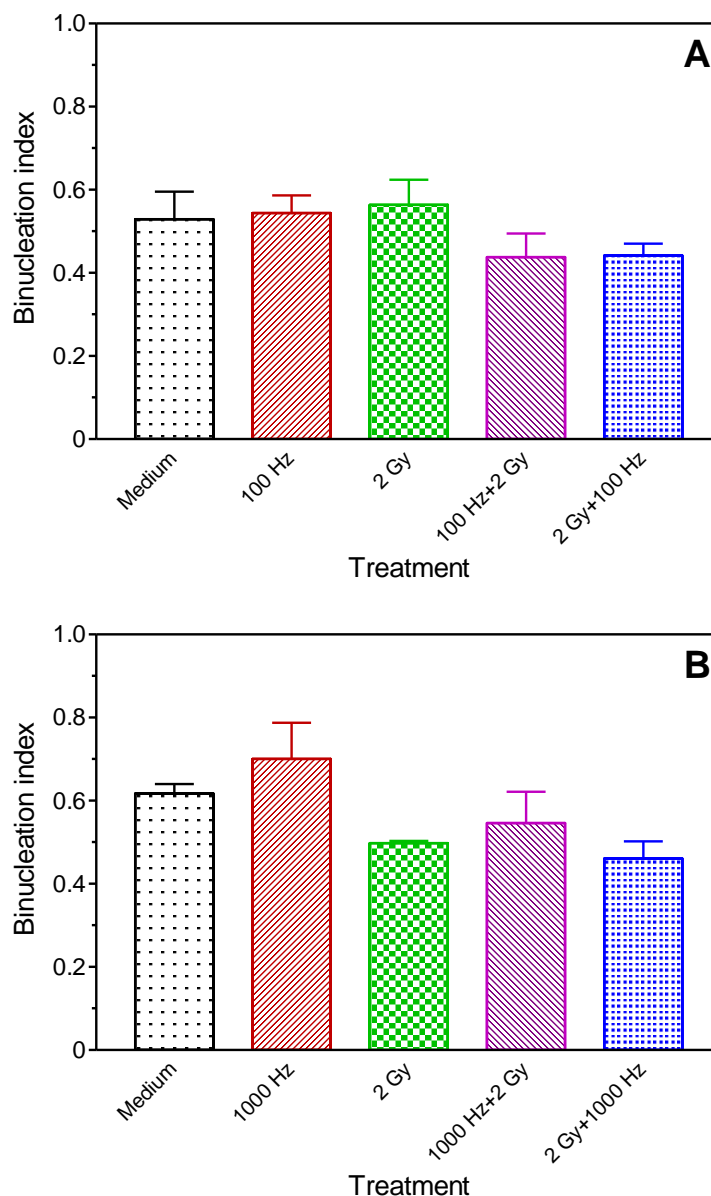


Figure 3.24: Binucleation index in the melanoma cell line, MeWo, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field.

While a 2-Gy of X-rays treatment alone or combined treatment with the 1000-Hz modulated RFF resulted in an expected decline in binucleation, exposure to the RFF alone increased cell proliferation by more than 10% (Figure 3.24B).

Be11

Exposure of Be11 cells to the 100-Hz and 1000-Hz modulated RFF resulted in ~2.0- and ~4.0-fold increases in micronucleus yield compared to background, respectively (Figure 3.25).

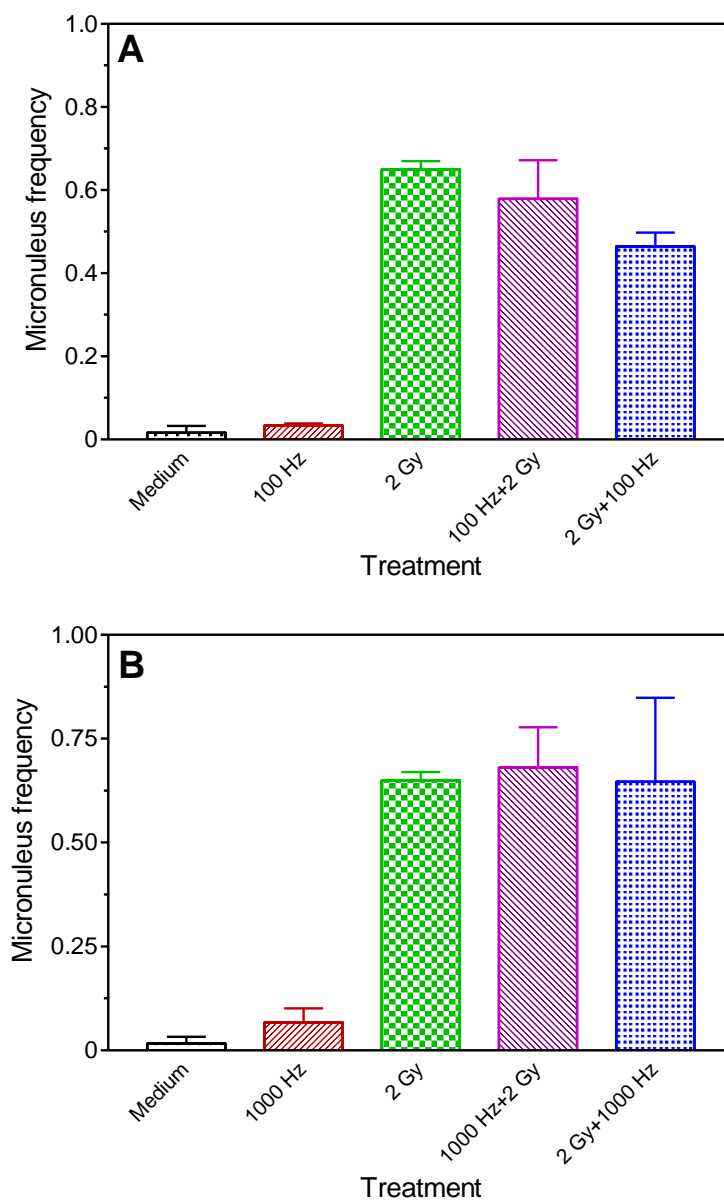


Figure 3.25: Micronucleus yield in the melanoma cell line, Be11, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field.

Combination treatment with the 100-Hz modulated RFF prior to or after 2 Gy of X-rays led to ~24% and ~30% reduction in micronucleus yield, respectively (Figure 3.25A). On the other hand, the 1000-Hz modulated RFF had no effect on radiation induced micronucleus frequency (Figure 3.25B).

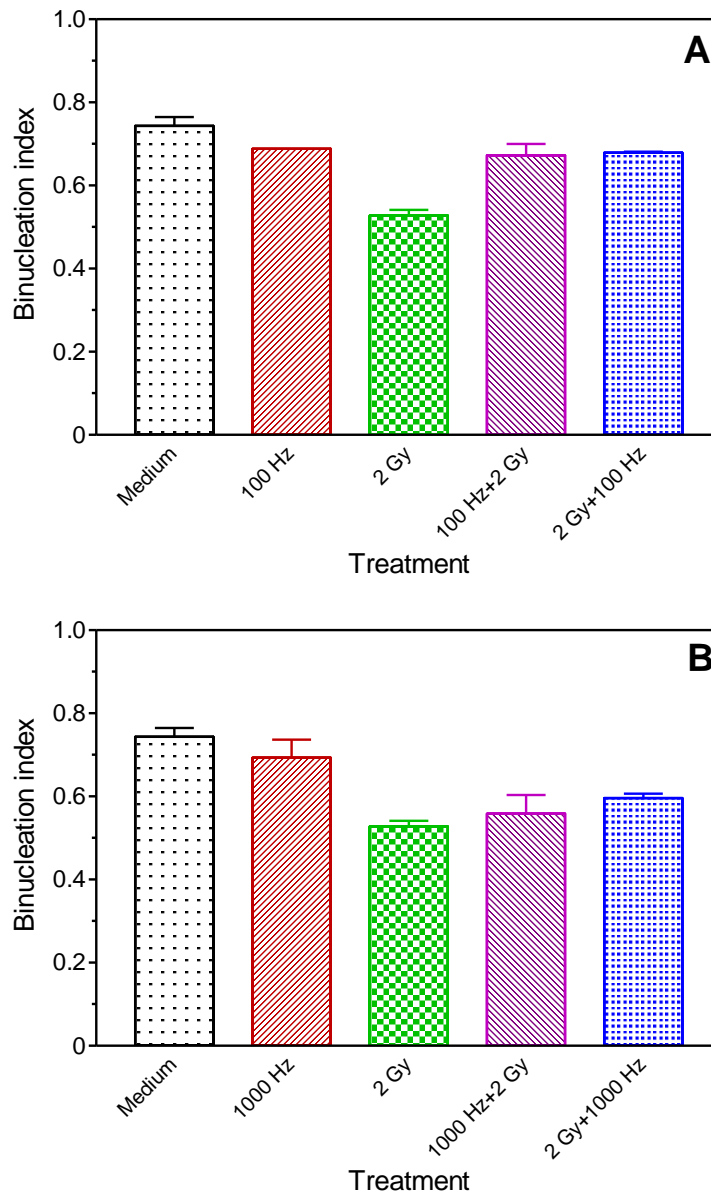


Figure 3.26: Binucleation index in the melanoma cell line, Be11, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field.

A slight reduction in binucleation was seen when cells were exposed to the 100-Hz or 1000-Hz modulated RFF alone (Figure 3.26). The decrease in binucleation at 2 Gy was reversed to levels close to those in background (medium and RFF control), when cells were concomitantly treated with the 100-Hz modulated RFF (Figure 3.26A). A similar, but less pronounced, recovery in binucleation was observed when cells were treated with a combination of X-rays and the 1000-Hz modulated RFF (Figure 3.26B).

L132

For the normal lung fibroblasts, L132, exposure to 100-Hz modulated RFF alone increased micronucleus yield, relative to background (Figure 3.27A). Combined treatment with this field led to up to 1.8-fold increase in micronucleus yield at 2 Gy, with the RFF exposure after X-ray irradiation being more damaging.

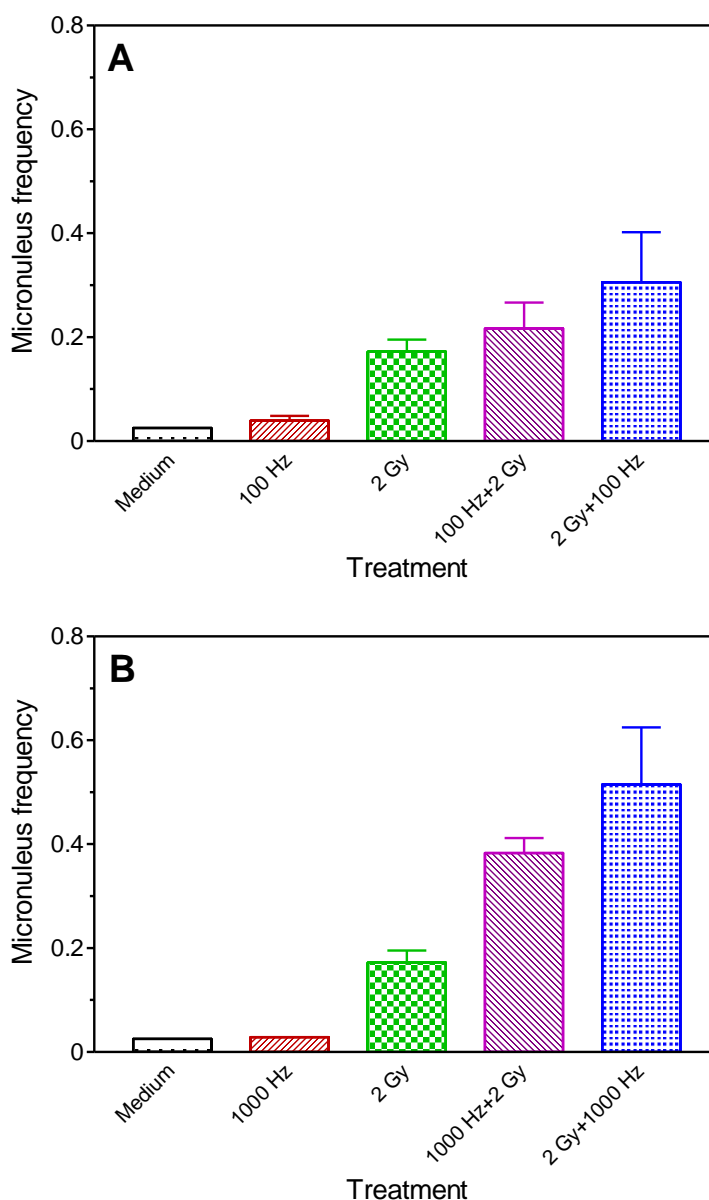


Figure 3.27: Micronucleus yield in the normal lung fibroblasts, L132, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field.

Treatment with the 1000-Hz modulated RFF alone had no impact on the background level of micronuclei (Figure 3.27B). As in the case of the 100-Hz modulated RFF, combination treatment with the 1000-Hz modulated RFF resulted in an up to 3.3-fold increase in micronucleus yield, with RFF exposure after 2 Gy of X-rays being more potent (Figure 3.27B).

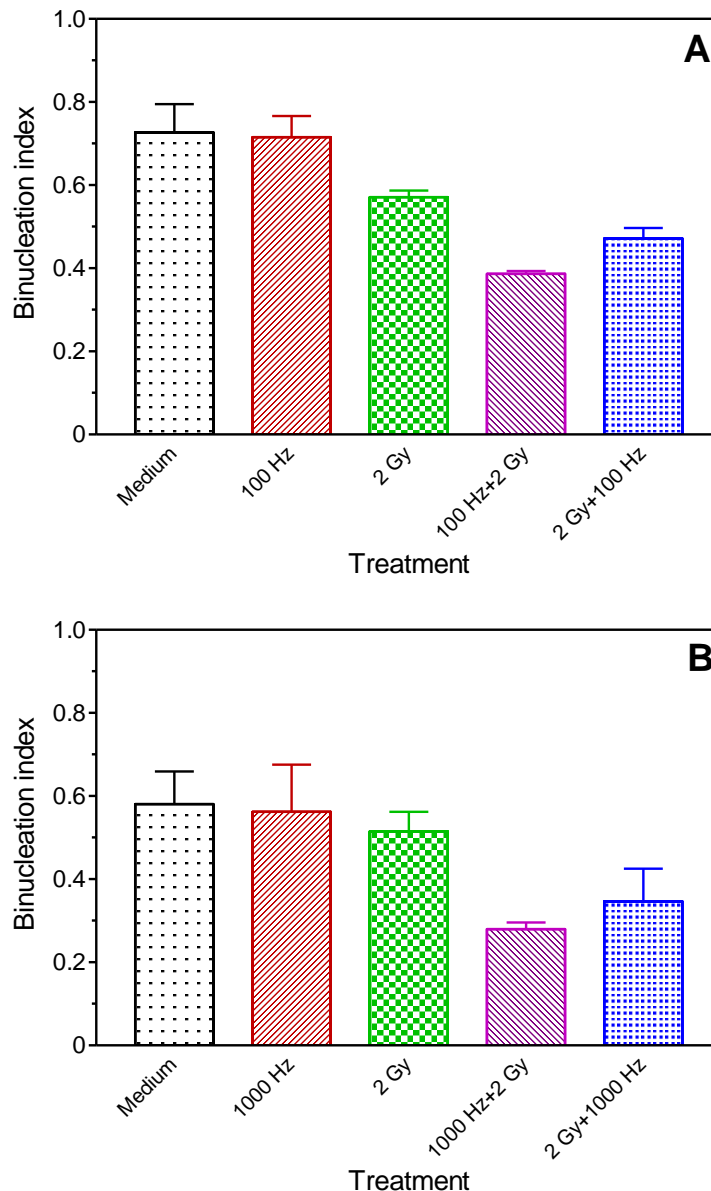


Figure 3.28: Binucleation index in the normal lung fibroblasts, L132, after treatment with 2 Gy of X-rays alone or in combination with a: (A) 100-Hz and (B) 1000-Hz modulated radiofrequency field.

Data for binucleation in the L132 cell line are presented in Figure 3.28. No effect on binucleation is apparent when cells were exposed to either the 100-Hz or 1000-Hz modulated RFF alone. Combined treatment with these radiofrequency fields reduced binucleation, relative to that obtained for the 2 Gy only treatment, with RFF exposure after X-ray irradiation being less potent in arresting cell proliferation.

Treatment induced modifying factors, based on micronucleus yield, as described in Section 2.11, are presented in Table 3.16. In the DU145 and Be11 cell lines, combining radiofrequency fields with X-ray irradiation either had no effect ($MF \approx 1.0$) or resulted in small reductions ($MF > 1.0$) in micronucleus yield. Similar treatment of the MeWo and L132 cell lines led to ~1.14 to 3.33-fold enhancement ($MF < 1.0$) in micronucleus frequency.

Table 3.16: Modifying factors (MF), relative to X-ray treatment alone, derived from the micronucleus frequency presented in Figures 3.21, 3.23, 3.25, and 3.7 for the DU145, MeWo, Be11, and L132 cell lines, respectively, as described in Section 2.11. Errors were calculated using error propagation formulae for ratios.

Treatment	DU145	MeWo	Be11	L132
100 Hz+2 Gy	1.07 ± 0.19	0.88 ± 0.11	1.16 ± 0.23	0.83 ± 0.26
2 Gy+100 Hz	0.99 ± 0.25	0.79 ± 0.11	1.47 ± 0.19	0.55 ± 0.22
1000 Hz+2 Gy	0.96 ± 0.16	0.53 ± 0.07	1.03 ± 0.18	0.41 ± 0.07
2 Gy+1000 Hz	1.21 ± 0.17	0.62 ± 0.08	1.10 ± 0.40	0.30 ± 0.08

3.10. Relationship between Radiosensitivity and Micronucleus Yield

To assess whether the radiosensitivity modifying effects seen when cells were concomitantly treated with radiofrequency fields and X-rays are influenced by changes in the level of DNA damage, the modifying factors derived from micronucleus yield were plotted against those determined from clonogenic cell survival. Figure 3.29A shows a weak trend [$Y=(0.36\pm 0.35)X+(0.49\pm 0.47)$; $R^2 = 0.1532$; $P = 0.3376$] where cell lines that are more radiosensitised produce lower yields of micronuclei, following combined treatment with the 100-Hz modulated RFF. Exclusion of the Be11 cell line which seems to be an outlier yields a strong correlation [$Y=(-1.08\pm 0.22)X+(2.12\pm 0.25)$; $R^2 = 0.8597$; $P = 0.0078$; Figure 3.29A; blue line] between the RFF induced radiosensitisation and DNA damage, whereby highly radiosensitised cell lines tend to express elevated levels ($MF < 1.0$) of micronuclei frequency.

A similarly weak correlation [$Y=(-0.15\pm 0.19)X+(1.05\pm 0.38)$; $R^2 = 0.0903$; $P = 0.4695$] was found for radiosensitisation and micronucleus yield for the combined treatment with the 1000-Hz modulated RFF, with more radiosensitised cell lines expressing higher levels of micronuclei (Figure 3.29B). Again, exclusion of the Be11 cell line, strengthens this correlation [$Y=(-0.45\pm 0.13)X+(1.42\pm 0.22)$; $R^2 = 0.7691$; $P = 0.0218$; Figure 3.29B; blue line].

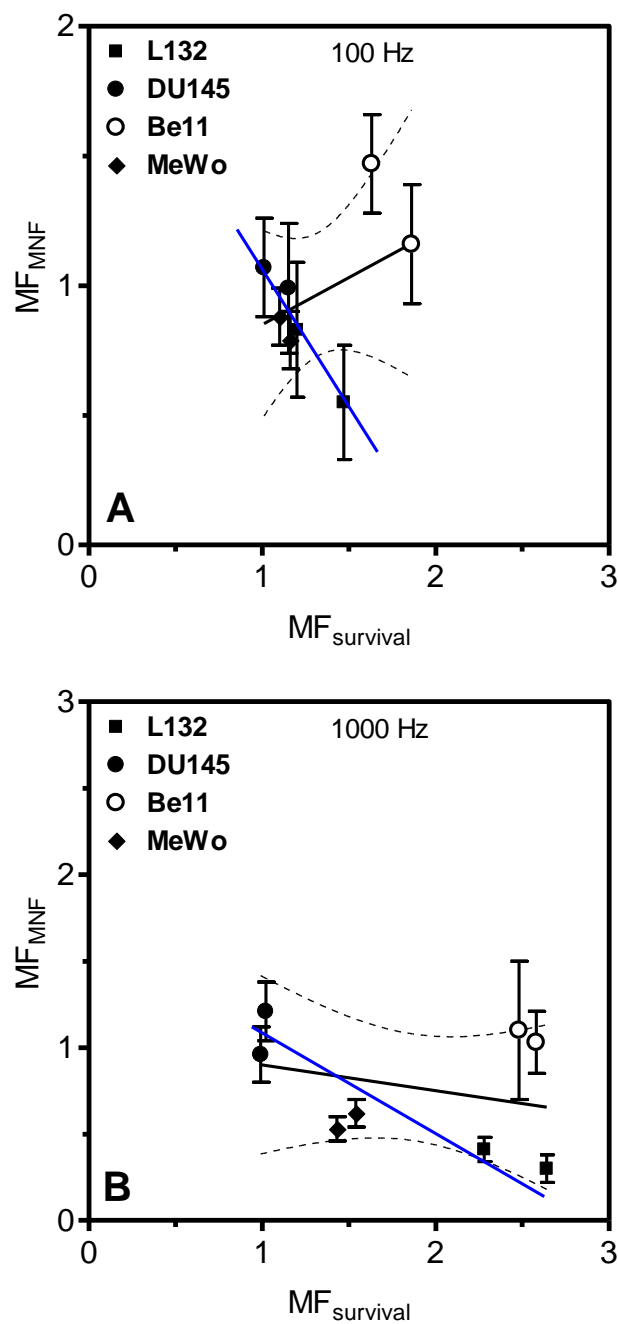


Figure 3.29: Plot of modifying factors from micronucleus frequency (MNF) at 2 Gy as a function of modifying factors from surviving fraction at 2 Gy for 4 cell lines: (A) combined treatment with 100-Hz modulated RFF and (B) combined treatment with 1000-Hz modulated RFF. Dashed lines represent the 95% confidence interval.

As described in Section 2.11, the modifying factors derived from binucleation indices are presented in Table 3.17. A combined treatment of the MeWo and L132 cell lines with radiofrequency fields and X-ray irradiation either had no effect ($MF \approx 1.0$) or

resulted in small reductions ($MF > 1.0$) in binucleation. Similar treatment of the DU145 and Be11 cell lines resulted in ~1.1 to 1.4-fold enhancement ($MF < 1.0$) in cell proliferation.

Table 3.17: Modifying factors (MF), relative to X-ray treatment alone, derived from the binucleation indices presented in Figures 3.22, 3.24, 3.26, and 3.8 for the DU145, MeWo, Be11, and L132 cell lines, respectively, as described in Section 2.11. Errors were calculated using error propagation formulae for ratios.

Treatment	DU145	MeWo	Be11	L132
100 Hz+2 Gy	0.91 ± 0.07	1.32 ± 0.28	0.73 ± 0.04	1.46 ± 0.18
2 Gy+100 Hz	0.84 ± 0.06	1.32 ± 0.24	0.72 ± 0.04	1.20 ± 0.16
1000 Hz+2 Gy	0.80 ± 0.08	1.04 ± 0.19	0.88 ± 0.09	1.78 ± 0.48
2 Gy+1000 Hz	0.81 ± 0.06	1.23 ± 0.19	0.76 ± 0.07	1.45 ± 0.49

3.11. Relationship between Radiosensitivity and Binucleation

The role of cell proliferation in the modulation of radiosensitivity by radiofrequency fields was assessed by plotting modifying factors derived from binucleation indices against those derived from clonogenic cell survival. Figure 3.30A demonstrates a weak trend [$Y=(-0.52\pm 0.34)X+(1.75\pm 0.46)$; $R^2 = 0.2786$; $P = 0.1788$] where an increase in radiosensitisation is mirrored by enhanced ($MF < 1.0$) binucleation, after combined treatment with the 100-Hz modulated RFF. A similarly weak, but reversed trend [$Y=(0.20\pm 0.20)X+(0.72\pm 0.39)$; $R^2 = 0.1421$; $P = 0.3573$] emerged for the 1000-Hz modulated RFF combined treatment (Figure 3.30B). However, as in the case of

micronucleus yield, exclusion of the Be11 cell line strengthens the correlation [$Y=(0.51\pm 0.13)X+(0.35\pm 0.23)$; $R^2 = 0.7976$; $P = 0.0165$; Figure 3.30B; blue line] between the RFF induced radiosensitisation and cell proliferation, where more radiosensitised cell lines tend to have reduced ($MF > 1.0$) binucleation.

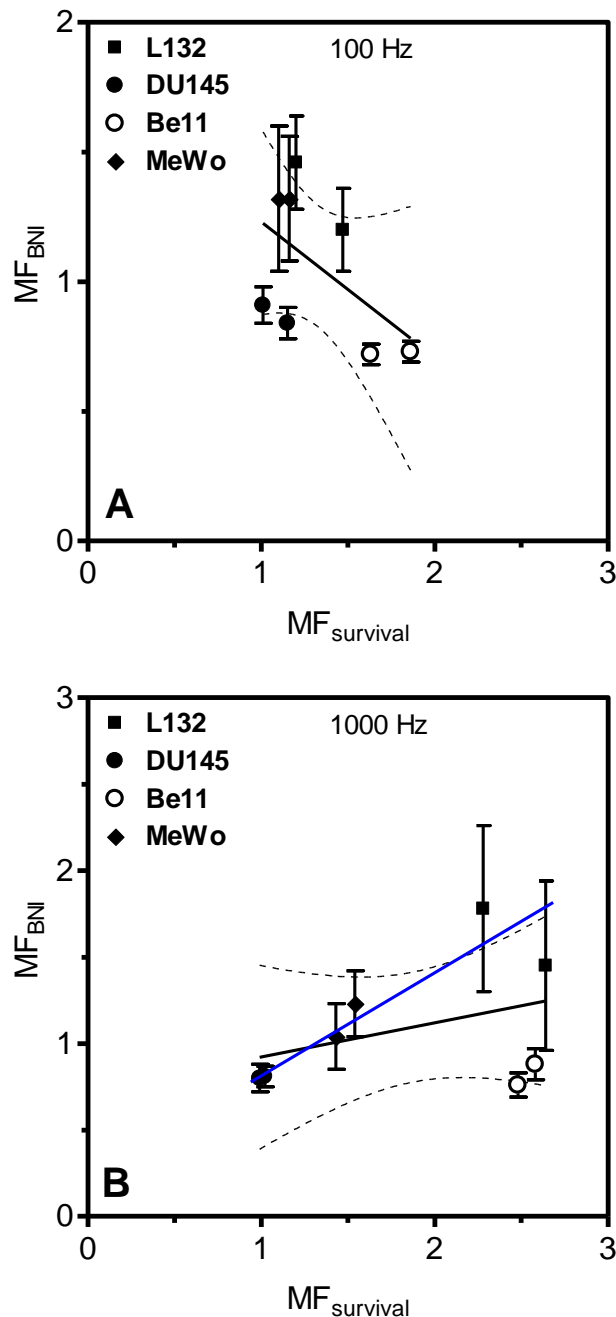


Figure 3.30: Plot of modifying factors from binucleation index (BNI) at 2 Gy of X-rays as a function of modifying factors from surviving fraction at 2 Gy for 4 cell lines: (A) combined treatment with 100-Hz modulated RFF and (B) combined treatment with 1000-Hz modulated RFF. Dashed lines represent the 95% confidence interval.

CHAPTER 4

4. Discussion

Radiation therapy (RT) is considered the first line of treatment for most superficial cancers, as these malignancies tend to respond well to radiation. The use of hypofractionated treatment may be beneficial for certain tumours, but this poses a normal tissue toxicity challenge as hypofractionation can cause severe side-effects from which the patient may not be able to recover. A possible approach to circumvent this challenge is the use of radiation modifying agents that can potentiate the tumour inactivating effects of ionising radiation and, therefore, lead to a reduction of radiation dose and normal tissue toxicity. Chemotherapeutic drugs are widely used in conjunction with radiotherapy, but do induce severe undesirable systemic effects (Azim et al., 2011; Palumbo et al., 2013; Alarid-Escudero et al., 2017).

Although magnetic fields have long been suggested as potential enhancers of radiation effects, only a handful of studies have been reported (Miyakoshi et al., 1999; Ding et al., 2000; Artacho-Cordón et al., 2013 for review). Studies on the combined biological effects of radiofrequency fields (RFF) and ionising radiation are virtually non-existent. The use of RFF as an adjuvant to radiotherapy may be beneficial, as they have been shown to exhibit *in vitro* radiosensitising and radioprotective effects on malignant and normal cells, respectively, with the possibility of a significant dose reduction (Chinhengo et al., 2018; 2019). However, there is a need to understand the potential mechanisms by which these RFF influence radiosensitivity so that they can be employed efficiently as radiotherapy modulators. The main goal of radiotherapy is to kill tumour cells and spare normal tissue, and a good modifying agent would be one

that sensitises the tumour to radiation while protecting the normal tissue. Here, the clonogenic cell survival assay was used as a measure of radiosensitivity. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), superoxide dismutase (SOD) and micronucleus assays were used to assess the possible mechanisms by which RFF influence the radiosensitivity of cells. The findings reported herein are intended to validate my initial observations (Chinhengo et al., 2018; 2019), and to assess candidate factors underlying the radiomodulatory effects of radiofrequency fields.

4.1. Intrinsic Radiosensitivity and Radiomodulatory Effect of Radiofrequency Fields

This study sought to compare intrinsic radiosensitivity to RFF modulated radiosensitivity, using the p53 mutant, human malignant melanoma MeWo cell line; the p53 wild-type, human melanoma Be11 cell line; the p53 mutant, human prostate cancer DU145 cell line; and the p53 wild-type, human normal lung epithelial L132 cell line. The intrinsic radiosensitivity data summarised in Table 3.10 show the DU145 to be the most radioresistant cell line and MeWo the most radiosensitive, giving a rank order of increasing radiosensitivity of DU145 → L132 → Be11 → MeWo, when the cell survival parameters SF_2 , SF_6 , and \bar{D} were collectively taken into account. Within the limits of experimental uncertainty, the SF_2 -values obtained here are consistent with those reported previously reported for the DU145 (Slabbert et al., 1996; Maleka et al., 2015), L132 (Roos et al., 2000), Be11 (Binder et al., 2000; Akudugu et al., 2004a), and MeWo (Binder et al., 2000) cell lines, indicating no unusual radiation response.

As stated before, an appropriate radiomodulator is one that is non-toxic on its own. These data demonstrate that cellular exposure to radiofrequency fields, irrespective of frequency, does not have a notable cytotoxic effect, as the plating efficiency of the cell line remained virtually unchanged (Table 3.1). This is not surprising, as several other studies have not demonstrated cytotoxic effects in a variety of cellular systems even at extremely high radiofrequency fields (Hirose et al., 2006; Lantow et al., 2006; Joubert et al., 2007). As such, radiofrequency fields could, therefore, find application as efficient radiation modulators in radiotherapy or radiation protection.

In this investigation, combined treatment with the 100-Hz modulated RFF and X-rays does not markedly affect the radiosensitivity ranking of the cell lines (Table 3.10). Pre- and post-exposure to 100 Hz only slightly altered radiosensitivity, with DU145 and MeWo the most radioresistant and most radiosensitive cell lines, respectively, for the most part. The exception being when cells were pre-exposed to 100 Hz, which rendered Be11 the most radiosensitive cell line. Pre- and post-exposure to the 1000-Hz modulated RFF, however, augmented the radiosensitivity of the Be11 and L132 cell lines significantly more than the DU145 and MeWo cell lines, making Be11 and L132 more radiosensitive than MeWo, with DU145 the most radioresistant in all treatments. The marked radiosensitisation in the human lung fibroblasts (Table 3.9) contrasts the finding of the initial study, using Chinese hamster lung fibroblasts (V79), which showed that RFF can act as radioprotectors in normal cells (Chinhengo et al., 2018). This suggests that a potential application of the 1000-Hz modulated RFF as an adjuvant in the clinical setting might not be generally appropriate, as it may significantly aggravate radiation-induced normal tissue toxicity.

The disparity in radiomodulation by RFF seems to be partly influenced by the p53 status of the cell line. The p53 wild-type cell lines (L132 and Be11) were consistently more sensitised compared to the p53 mutant cell lines (MeWo and DU145), as is apparent from the modifying factors presented in Tables 3.3, 3.5, 3.7, and 3.9. The slight sensitisation of the p53 mutant cell lines used here and the radioprotection demonstrated in the apparently normal V79 cells in a previous study (Chinhengo et al., 2018) suggest that RFF influence radiosensitivity in ways that may be independent of p53 function. It is worth noting that p53 is also mutated and non-functional in V79 cells (Chaung et al., 1997). However, the significant sensitisation of p53 wild-type cell lines suggests that RFF might target the p53 survival pathway, influencing it to enhance radiosensitivity. This would contrast with the report by Hirose and colleagues suggesting that radiofrequency field in the GHz range do not affect p53 phosphorylation (Hirose et al., 2006). However, the effects of radiofrequency fields on macromolecules are largely frequency-dependent (Agulan et al., 2015). The fields used in the current study may enhance radiation-induced damage to macromolecules, but damaged cells that are p53 mutant may evade p53-mediated apoptosis, dying through other modes (Tait et al., 2014) or surviving, while their p53 wild-type counterparts are eliminated through a p53-mediated apoptotic process. This further emphasises the need for caution in a possible combination of RFF with ionising radiation in cancer therapy, as a significant component of cancers are p53 mutated and such mutations might sometimes infer gain of certain protective functions (Muller and Vousden, 2013). It is also possible that RFF exposure activates other genes in different survival pathways, the influence on survival depending on whether genes in the targeted pathway are functional or altered, rendering them dysfunctional. The

presence of a dysfunctional gene in a survival pathway brings discontinuity to a pathway.

The influence of RFF seems to be cell line and frequency dependent (Chinhengo et al., 2018), more so at higher radiation doses. All the radiofrequency fields evaluated here affect the radiosensitivity to more or less the same extent. DU145 is sensitised the most by the 2000-Hz modulated field at high radiation doses, with the 100-, 1000- and 4000-Hz modulated fields having the same influence on the cell line. MeWo, Be11 and L132 are sensitised the most by 1000-Hz modulated field in combination with high radiation doses, with the 100-, 2000- and 4000-Hz having the same influence on these cell lines. These results suggest that any potential use of RFF, as an adjuvant to radiation therapy, needs to be regulated and guided by the characteristics of each cancer e.g. type and p53 status. This, especially that RFF modulated at higher frequencies tended to promote cell survival at the higher radiation dose of 6 Gy (Figures 3.5 – 3.8). Such radioprotection by fields modulated at high frequency could have significant implications for the potential use of RFF in hypofractionation settings where large fractional doses are employed.

4.2. Effect of Radiofrequency Fields on Radiation-Induced Changes in Metabolic Activity and its Relation to Overall Cell Survival.

To determine the effect of RFF on X-ray induced changes in metabolic activity, relative metabolic activities were determined at 30 min and 18 h, and used to derive modifying factors, as described in Section 2.9. The expectation was for the more radiosensitised cell lines to exhibit the relatively higher metabolic activity (increased metabolic activity,

represented here by $MF_{MTT} < 1.0$) and vice versa, as there is evidence to suggest that reduced metabolic rates lead to radioresistance (Luk and Sutherland, 1987; Heller and Raaphorst, 1993; Moeller et al., 2005). The data in Figure 3.13 (especially for the 100-Hz modulated RFF) seem to reflect the opposite, with a trend towards cell lines that were more radiosensitised by RFF exposure showing the larger reductions in metabolic activity in relation to radiation treatment alone, regardless of treatment sequence. There is no link between intrinsic radiosensitivity and metabolic rate in untreated cell cultures, suggesting that the observed trend may be due to treatment related alterations in metabolic rate. Within the first hour of treatment, changes in metabolic activity may be triggered to support processes like DNA repair.

This relationship between metabolic rate and radiosensitisation persisted, especially for the cancer cell lines, when metabolic activity was assessed at a much later time point (Figure 3.14). At 18 h post-treatment, increased cellular metabolism may be thought as supporting processes, such as proliferation, while a reduction might signal a cell cycle arrest. The phenomenon of radiofrequency field-induced increase in metabolic activity may be due to the claim that electromagnetic fields can cause cells to move from inactive phases of the cell cycle to the more active G2/M phases, with associated elevations in proliferation (Velizarov et al., 1999; Sun et al., 2009). An enhanced proliferation could make cells vulnerable to radiation damage and lead to a high level of radiosensitisation. However, the effects elicited by electromagnetic fields seem to be much dependent on cell type. While high frequency RFF have been shown to induce significant levels of cell cycle arrest in neuronal cells (Buttiglione et al., 2007), no such effects were demonstrated in fibroblasts and glioma cells (Higashikubo et al.,

2001). These differential influences of electromagnetic fields can lead to either radiosensitisation, radioprotection, or no effect on cellular radiosensitivity.

These results suggest that metabolic activity may be a determinant of long-term survival. Assessment of how radiofrequency fields affect radiation-induced cell killing in an expanded panel of cell lines could clarify the importance of p53 status in radiomodulation by RFF.

4.3. Effect of Radiofrequency Fields on Radiation-Induced Changes in Superoxide Dismutase (SOD) Activity and its Relation to Overall Cell Survival.

Reactive oxygen species (ROS) plays an important role in cellular signalling and has a dual role in cancer. ROS can facilitate cancer cell proliferation, survival, and adaptation to hypoxia while, on the other hand, ROS can cause oxidative stress, leading to cell death (López-Lózaró, 2007; Pan et al., 2009; Gupta et al., 2012). Reactive oxygen species is produced because of increased metabolic activity in cells and the dysfunction of mitochondria. Electromagnetic fields have been reported to influence the production of ROS in cells (Lai and Singh, 2010; Morabito et al., 2010; Kovacic and Somanathan, 2010), and its concentration may either enhance survival of the cells or cause macromolecule damages leading to cell death. Superoxide dismutase (SOD) is one of the enzymes responsible for removing or converting reactive oxygen species to less harmful compounds and the concentration of SOD is relative to the ROS levels in the cell. In this work, the effect of a 1000-Hz modulated radiofrequency field and 6 Gy of X-rays on the SOD concentration was assessed and deemed to signify the ROS levels in response to treatment.

Unlike changes in metabolic activity that seemed to be correlated with radiomodulatory effects of RFF exposure across cell lines, no link was apparent between alterations in SOD activity and RFF-mediated radiosensitisation (Figure 3.20). This finding is not surprising, given the multifunctional features of reactive oxygen species (López-Lózaró, 2007; Pan et al., 2009; Gupta et al., 2012). Superoxide dismutase activity was only markedly reduced ($MF_{SOD} > 2.0$) in the p53 mutant DU145 and the p53 wild-type Be11 cells, when cells were pre-exposed to the radiofrequency field. No effect was apparent in the other treatments and cell lines, indicating that RFF-mediated changes in radiation-induced SOD activity do not appear to depend on p53 status or treatment sequence.

Although superoxide dismutase has been known to play a key role in regulating cellular metabolism (Sarsour et al., 2012), the current study has not identified a link between SOD and metabolic activity. The modes by which changes in SOD and metabolic activity may impact on radiomodulatory effects of radiofrequency fields appear to be unrelated.

4.4. Effect of Radiofrequency Fields on Radiation-Induced Micronucleus Frequency, and the Possible Impact on Cell Survival.

Reports on the capacity of radiofrequency fields at inducing micronuclei are mixed. While some studies do not demonstrate elevated levels of micronuclei in cellular systems following RFF exposure (Vijayalaxmi et al., 2001; Bisht et al., 2002; McNamee et al., 2002), others have shown enhanced micronucleus formation

(Koyama et al., 2003; 2004). The slight elevation in micronucleus formation seen in this study may be due to the much lower frequencies used.

From the summary of the micronucleus data presented in Figure 3.29, the melanoma cell line (Be11) is a clear outlier, being highly radiosensitised but exhibiting reduced levels of micronuclei ($MF_{MNF} > 1.0$). Low micronucleus yield can be attributed to the combination treatment causing such extensive damage to the cells that they die and, therefore, do not present as binucleated cells with micronuclei for assessment (Akudugu et al., 2000). The number of lethal lesions at 2 Gy is given as the negative natural logarithm of SF_2 (Bush and McMillan, 1993; Villa et al., 1994; Akudugu et al., 2000). The ability of a cell to survive a certain level of micronucleus yield can be expressed in terms of the number of micronuclei per lethal lesion (i.e. the number of micronuclei that would constitute a lethal event in the cell) (Akudugu et al., 2000). Here, the combination treatments resulted in 0.35 - 0.52 micronuclei per lethal lesion in the Be11 cell line, implying that these cells are more resistant to micronuclei-mediated death than the L132 cells (0.17 - 0.40 micronuclei per lethal lesion). For the DU145 and MeWo cells, the number of micronuclei per lethal lesion ranged from 0.80 to 1.00 and 0.69 to 1.14, respectively, indicating an even higher resistance to micronucleus lethality. Treatment-induced reduction in micronucleus yield is, therefore, not due to cell death.

A reduced micronucleus yield can also be a consequence of efficient DNA repair, as this would lead to fewer residual acentric fragments to present as micronuclei. In fact, Be11 cells have been shown to exhibit ~97% DNA double-strand break rejoining, 20 h after γ -ray irradiation (Theron et al., 2000; Akudugu et al., 2004a). Elsewhere, the

L132 cells which also show low numbers of micronuclei per lethal lesion but an RFF-induced increase in micronucleus yield, have been found to be equally repair proficient (Roos et al., 2000). However, the DU145 and MeWo cells which fall between the Be11 and L132 cells in terms of micronucleus yield are intrinsically less efficient in DNA double-strand break rejoining at ~88% (El-Awady et al., 2003) and ~81% (Theron et al., 2000), respectively. Exclusion of the Be11 cell line yields a strong frequency-independent correlation between RFF-mediated micronucleus yield and radiosensitisation, with the more radiosensitised cell lines showing increased micronucleus yield (Figure 3.29). The relationship between radiofrequency-mediated changes in micronucleus yield and DNA repair is, thus, not straightforward. However, these findings seem to suggest that radiofrequency exposure perturbs DNA damage repair processes, a phenomenon that could have important implication in radiotherapy and radiation protection.

4.5. Effect of Radiofrequency Fields on Radiation-Induced Changes in Binucleation.

The binucleation index can be used as an indicator of cell proliferation (Akudugu and Böhm, 2001; Akudugu et al., 2001; 2004b). A high binucleation index indicates a large proportion of cells progressing through a cell division, and vice versa. The impact of radiofrequency exposure on cell proliferation and cell cycle progression is not consistent. Although some studies have demonstrated changes in cell cycle progression and reductions in cell proliferation after extended exposure to RFF (Velizarov et al., 1999; Marinelli et al., 2004), others have failed to detect similar effects (Pacini et al., 2002; Gurisik et al., 2006; Sanchez et al., 2006). Based on the

binucleation data presented here, RFF exposure does not appear to have an impact on proliferation of DU145, MeWo, Be11, and L132 cells. The data presented in Figure 3.10 again shows the Be11 cell line as a possible outlier, which is highly radiosensitised but retains a high level of cell division ($MF_{\text{BNI}} < 1.0$). Elevated radiosensitivity in cells that continue to progress through a division after treatment may be related to an absence of a cell cycle block that might be required for adequate recovery. However, this cell line exhibits both G₁- and G₂-phase blocks at 12 and 16 h, respectively, while its p53 mutated counterpart (MeWo) shows only a G₂-phase block at about 18 h (Binder et al., 2000). Therefore, the elevated binucleation in Be11 cannot be explained by the inexistence of a cell cycle arrest. The increased radiosensitivity in Be11 can be attributed to existence of the G₁-phase block as arrests at this stage of the cell cycle are said to be responsible for high radiosensitivity (McIlwrath et al., 1994).

Considering only the DU145, MeWo, and L132 cell lines, cells that showed reduced binucleation ($MF_{\text{BNI}} > 1.0$) tended to be more radiosensitised. This may be due to the fact that cell cycle arrests in these cell lines after ~7 Gy irradiation have been noted to last as long as 40 h (Binder et al., 2000; Roos et al., 2000). It should be noted that except for the MeWo cell line (48 h), the micronucleus assay was terminated after 24 – 42 h, based on predetermined doubling times (Section 2.11). It is, therefore, likely that the binucleation indices reported here represent levels that correspond to incomplete re-entry of cells into cycling. Nonetheless, these experiments were performed at 2 Gy and the residence time of cells in phase blocks can be expected to be much shorter, as demonstrated by Su and Little almost three decades ago (Su and

Little, 1993). The finding that radiofrequencies may activate proliferation in certain cell types could be of significance to wound healing and needs to be further interrogated.

CHAPTER 5

5. Conclusion

Radiofrequency fields have the potential of modulating radiotherapy to improve tumour control. The data presented here show that radiofrequency fields are more efficient in modulating large fractional doses of X-rays and could find application in hypofractionated radiotherapy as adjuvants, especially for tumours with low alpha/beta ratios. This can have a significant positive impact on the management of patients with superficial tumours that may be resistant to low fractional doses of radiation. Radiofrequency fields modulate cellular radiosensitivity in a frequency- and cell type-dependent manner. Their effects on radiosensitivity also appear to be linked to p53 status. From the results reported here, cellular responses like metabolism, DNA damage processing (based on micronuclei formation), and proliferation (based on binucleation) seem to be underlying factors mediating the radiomodulatory effects of radiofrequency fields. No potential mediatory role was identified for reactive oxygen species.

Overall, there is more than one mechanism by which radiofrequency fields can modulate radiosensitivity, such as amplification of radiation-induced genotoxicity, cell cycle arrest, and disturbance of other cellular biochemical processes that lead to alteration of homeostasis. Other possible ways by which RFF affect radiosensitivity are, interfering with the synthesis and function of charged proteins in the cell leading to programmed cell death or premature cell ageing, perturbation of intracellular calcium ions which can trigger apoptotic or necrotic cell death, and/or modulating the expression of Bcl-2 family proteins. Given this complexity, a potential use of

radiofrequency fields as a non-invasive therapeutic modality would require standardisation to establish reproducibility. A more detailed understanding of how radiofrequency fields interact with ionising radiation would also be beneficial in the radiation protection field. However, a major limitation of the studies described herein is that irradiation of cell cultures with X-rays while they are being exposed to radiofrequency fields is not feasible. The possibility of truly exposing cells to X-rays and RFF simultaneously might result in even more pronounced radiomodulatory effects.

Possible Future Avenues

The importance of radiation modifiers in radiotherapy, radiation protection, and biological dosimetry cannot be overemphasised. When radiation modifiers are used in radiotherapy, the challenge is to obtain enhanced tumour response (potential radiosensitisation), while keeping normal tissue response unchanged or reduced (potential radioprotection). The extent of the biological effect of a given dose of ionising radiation (biological dosimetry) can be either reduced (in the case of the modifier being a radioprotector) or increased (in the case of the modifier being a radiosensitiser). The findings reported previously (Chinhengo et al., 2018; 2019), and in this study, demonstrate that radiofrequency fields could have desirable radiosensitising and radioprotective effects. The weakness of correlations, or lack thereof, between the radiomodulatory effects of radiofrequency fields and cellular metabolic activity, superoxide dismutase activity, and DNA damage (based on micronuclei induction) profiles, and p53 status is most likely due to the small number of cell lines studied. Conducting similar studies on an expanded panel of cell lines (both malignant and normal), and possibly, on peripheral blood lymphocytes (with assays other than the colony forming assay) could provide further insight into understanding the modes by which radiofrequency fields affect cellular responses to ionising radiation.

Specifically, the following avenues could be explored:

1. Assessment of the following after ionising radiation/radiofrequency exposure, using more efficient techniques like flow cytometry:
 - DNA damage processing capacity (e.g. γ H2AX foci assay).

- superoxide dismutase activity (e.g. using hydroethidine and 2',7'-dichlorofluorescein).
- metabolic activity (e.g. fluorescein diacetate activity assay).
- p53 activity (e.g. via detection of p21).
- cell cycle progression (e.g. measurement of DNA content).

2. Repeat of (1) for:

- radiofrequency field exposure longer than 30 min.
- more than one fraction of radiofrequency field exposure.
- more than one fraction of ionising radiation.

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Appendix A: Publications

Published Abstracts

1. Chinhengo A, Serafin AM, Akudugu JM. Evaluation of cellular sensitivity to split radiation dose and a combination of a single radiation dose and electromagnetic field exposure. *Physica Medica: Eur J Med Phys* 41:S9, 2017; DOI:10.1016/S1120-1797(17)30295-8

Conference Contributions

1. Chinhengo A, Serafin A, Akudugu JM. Radiofrequency waves preferentially enhance *in vitro* cellular radiosensitivity to large fractional doses. 63rd Academic Year Day, Faculty of Medicine and Health Sciences, University of Stellenbosch, Tygerberg, South Africa, August 2019 (oral).
2. Chinhengo A, Serafin A, Akudugu JM. Effects of low and intermediate electromagnetic waves on radiosensitivity of normal and cancer cells *in vitro*. 62nd Academic Year Day, Faculty of Medicine and Health Sciences, University of Stellenbosch, Tygerberg, South Africa, August 2018 (oral).
3. Chinhengo A, Serafin A, Akudugu JM. Evaluation of cellular sensitivity to a split radiation dose and a combination of a single radiation dose and electromagnetic field exposure. SAAPMB Congress, Durban, South Africa, September 2017 (oral).
4. Chinhengo A, Serafin A, Akudugu JM. Evaluation of cellular sensitivity to a split radiation dose and a combination of a single radiation dose and electromagnetic field exposure. 61st Academic Year Day, Faculty of Medicine and Health Sciences, University of Stellenbosch, Tygerberg, South Africa, August 2017 (oral).

5. Chinhengo A, Serafin A, Akudugu JM. Evaluation of cellular sensitivity to a split radiation dose and a combination of a single radiation dose and electromagnetic field exposure. 18th SASMO/SASCRO Congress, Johannesburg, South Africa, August 2017 (oral).

Appendix B: Original Ethics Exemption



Health Research Ethics Committee (HREC)

Approval Notice

New Application

15/05/2018

Project ID :1429

HREC Reference #: S17/10/207

Title: EVALUATION OF THE EFFECT OF RADIO FREQUENCY ELECTROMAGNETIC WAVES ON RADIOSENSITIVITY

Dear Ms Angela Chinhengo,

The **Response to Stipulations** received on 11/05/2018 09:28 was reviewed by members of **Health Research Ethics Committee 2 (HREC2)** via **expedited** review procedures on 15/05/2018 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: **This project has approval for 12 months from the date of this letter.**

Please remember to use your **Project ID [1429]** on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see [Forms and Instructions](#) on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

The HREC will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: <https://www.westerncape.gov.za/general-publication/health-research-approval-process>. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: [Forms and Instructions](#) on our HREC website <https://applyethics.sun.ac.za/ProjectView/Index/1429>

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Francis Masiye ,

HREC Coordinator,

Health Research Ethics Committee 2 (HREC2).

National Health Research Ethics Council (NHREC) Registration Number:

REC-130408-012 (HREC1) REC-230208-010 (HREC2)

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:
IRB0005240 (HREC1)-IRB0005239 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the [World Medical Association \(2013\). Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects](#); the [South African Department of Health \(2006\). Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa \(2nd edition\)](#); as well as the Department of Health (2015). Ethics in Health Research: Principles, Processes and Structures (2nd edition).

The Health Research Ethics Committee reviews research involving human subjects conducted or supported by the Department of Health and Human Services, or other federal departments or agencies that apply the Federal Policy for the Protection of Human Subjects to such research (United States Code of Federal Regulations Title 45 Part 46); and/or clinical investigations regulated by the Food and Drug Administration (FDA) of the Department of Health and Human Services.

Appendix C: Extended Ethics Exemption



15/05/2019

Project ID: 1429

Ethics Reference #: S17/10/207

Title: EVALUATION OF THE EFFECT OF RADIO FREQUENCY ELECTROMAGNETIC WAVES ON RADIOSENSITIVITY EVALUATION OF THE EFFECT OF RADIO FREQUENCY ELECTROMAGNETIC WAVES ON RADIOSENSITIVITY

Dear Ms Angela Chinhengo,

Your request for annual renewal of ethics approval submitted on 30 April 2019 refers. The Health Research Ethics Committee (HREC) reviewed the annual progress report you submitted and approved it.

The approval of this project is extended for a further year.

Approval date: 15 May 2019

Expiry date: 14 May 2020

Kindly be reminded to submit progress reports two (2) months before expiry date.

Where to submit any documentation

Kindly note that the HREC uses an electronic ethics review management system, *Infonetica*, to manage ethics applications and ethics review process. To submit any documentation to HREC, please click on the following link: <https://applyethics.sun.ac.za>.

Please remember to use your **Project ID [1429]** and **Ethics Reference Number [S17/10/207]** on any documents or correspondence with the HREC concerning your research protocol.

Yours sincerely,

Mr. Francis Masiye,

HREC Coordinator,

Health Research Ethics Committee 2 (HREC2).

*National Health Research Ethics Council (NHREC) Registration Number:
REC-130408-012 (HREC1)-REC-230208-010 (HREC2)*

*Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:
IRB0005240 (HREC1)-IRB0005239 (HREC2)*

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