بسم الله الرحمن الرحيم

Sudan Academy of Sciences, SAS
Atomic Energy Council

Radiobiological Characterization of two Photon-Beam Energies 6and15MV used in Radiotherapy from Linear Accelerator

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A Thesis Submitted to Sudan Academy of Sciences in Fulfillment of the Requirements for Master of Science in Biochemistry

Supervisor:

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DEDICATION

To my Uncles
To my

F

R

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E

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D

S

To my
Brothers
With endless love

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Abstract

The main objective of this study is to perform radiobiological characterization of two different photon beam energies, 6MVand15MV, from linear accelerator used in radiotherapy, with special regard to late effects of radiation. Two end-points; namely cell survival and micronucleus induction were used for the characterization. Chinese hamster V79 lung fibroblast cell line to prepare cell culture and to perform the *invetro* experiments. Chromosomes number was counted and found to be 22 chromosomes per cell; this result is in complete agreement with the expected 11pairs of chromosomes representing the genome of this species. Cells were kept in confluent growth for two days and then exposed to two photon beam energies, 6 and 15MV respectively. Different dose rates were used for the two beam energies, 0.25, 0.5, 1.0, 2.0, 4.0, 7.0 Gy. Cells were counted immediately after irradiation and reseeded, the seeded number of cells was calculated according to the dose rate used. Another set of unirradiated cells treated the same as the experimental set was used as a control group. The plating efficiency (PE) was calculated for the control group, then cells were incubated at 37ºC for 6 days to construct the survival curve, five samples were counted per dose and the mean was calculated. The two survival curves are similar for photon beam energies (6 and 15 MV) and the surviving fraction was decreased with dose rate. The two curves showed similar values of α and β parameters, this result is expected for the same radiation type (X-ray). For the micronuclei assay three samples for each dose were seeded and incubated at 37ºC for 24 hours then Cytochalasin-B was added to block cells in cytokinesis phase of the mitosis. The micronuclei number was counted and plotted with dose. A significant positive correlation was found between dose and micronuclei frequency (P=0.00), moreover, the micronuclei frequency is relatively higher with 15MV compared with 6MV energy. this indicates the presence of low dose of photoneutrons produced by high energy photon beam. Because of this effect more evaluation of radiation protection may be needed in radiotherapy patients especially when applying high energy photons.

الخـــــلاصـــــة

تعيين الخصائص البيولوجية والإشعاعية لحزمتين من الفوتونات ذات طاقة مختلفة تنتج من المسرع الخطى وتستخدم في العلاج الاشعاعى للأورام

أجريت هذه الدراسة بغرض تعيين الخصائص البيولوجية والإشعاعية لحزمتين من الفوتونات ذات طاقتين مختلفتين (6 و15 ميغافولت) تنتج من ال مسرع الخطى وتستخدم في العلاج الإشعاعى للأورام لمعرفة مدى التأثير المتأخرللإشعاع وذلك من خلال دراسة ناتجين نهائيين لمنحنى البقاء والنويات الصغيرة (Survival Curve and Micronuclei) على نوع معين من خلايا القوارض (V79Rodent cell line) .تم تحديد عدد الصبغيات (Karyotyping) في الخلايا المستخدمة ووجد أن العدد المثالي 22 كروموسوم لكل خلية بعد حساب 60 خلية. لحساب منحنى البقاء والنويات الصغيرة تم تزريع الخلايا في وسط غذائي مناسب وحضانتها لفترة يومين ومن ثم تعريضها للحزم الفوتونية الإشعاعية بمعدل جرعات من 0.25, 0.5, 1.0, 2.0, 4.0الى 7.0 Gy وبعد ذلك تم ت إزالة الوسط الغذائي وعد الخلايا بواسطة عداد خلايا (cell coulter counter) وإعادة تزريعها بأعداد معينة لكل جرعة إشعاعية فى وسط غذائى جديد تم ت حضانة الخلايا في درجة حرارة 37ºC لمدة 6أيام لتحديد منحنى البقاء كما تم تزريع جزء من الخلايا لكل جرعة لتحديد النويات الصغيرة وتم ت حضانتها لمدة 24 ساعة ثم إ ضافة مادة (Cytochalasin-B) لإيقاف الإنقسام الخلوي في مرحلة إنقسام السيتوبلازم (Cytokinesis) . تم عد النويات الصغيرة بمعدل 3000 خلية لكل جرعة و أخذ الوسط الحسابى لها وبمقارنتها بعينات

التحكم وجد أنها تزداد بزيادة الجرعة وبمقارنة 6 ميغافولت مع 15 ميغافولت وجد أن عدد النويات الصغيرة يزداد بزيادة طاقة الفوتون المستخدم مما يدل على وجود فوتونيوترون (Photoneutrons) تنتج من إستخدام فوتونات ذات طاقة عالية.

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Abbreviations

MeV	Mega electron Volt
IR	Ionizing Radiation
Gy	Gray
LINAC	Linear Accelerator
Rad	Radiation absorbed dose
W_R	Radiation weighting factor
Rem	Radiation equivalent for man
RBE	Relative Biological Effectiveness
OER	Oxygen Enhancement Ratio
SF	Surviving Fraction
PE	Plating Efficiency
MN	Micronuclei
ECC	European Code against Cancer.
EI	Education Intervention
EBRT	External Beam Radiotherapy
3D-CRT	Three-Dimensional Conformal Radiotherapy
IMRT	Intensity-modulated radiotherapy
NHL	Non-Hodgkin's Lymphoma.
CML	Chronic Myeloid Leukemia
NCCP	National Cancer Control Programme
RICK	Radiation isotope Centre Khartoum
AML	Acute Myelogenous Leukemia
MDS	Myelodysplastic Syndrome
CBMN	Cytokinesis-Block Micronuclei assay

LET	Linear Energy Transfer
AT	Ataxia Telangiectasia
FCS	Foetal Calf Serum
Z	Atomic number
GSTM1	Glutathione S-transferase M1
SIR	Standardized Incidence Ratio

Introduction

Sudan, the largest country in Africa by area, sits between North Africa, sub-saharan Africa, and the Middle East, with ethnic populations from all these regions and very diverse regional climates, Sudan could be considered as a microcosm of Africa. Over 80% of the population live in rural settings or are nomadic, presenting great challenges to any disease control initiative. Sudan first initiated a national cancer control programme (NCCP) in 1982 in association with the world health organization (WHO), and this was updated in the early 1990s based on the new WHO guidelines. This programme focuses on prevention, early detection, screening, improved diagnosis, treatment and palliative care. In 2000, based on hospitals data, cancer was found to be the third leading cause of death after malaria and viral pneumonia, accounting for 5% of all deaths. As from 1967 the Radiation and Isotopes Centre-Khartoum (RICK) started to operate as the only radiotherapy centre in Sudan. Based on statistics, nasopharyngeal carcinoma and non-Hodgkin's lymphoma (NHL) were the most common tumours in Sudanese males for the following 18 years after the establishment of RICK. In the last 20 years, chronic myeloid leukemia (CML) became the predominant cancer, while lymphomas remained the second most common cancer in men. In women, breast, cervical and ovarian cancers remained the three most common cancers over both time periods, but there was also an increase in the incidence of CML among women. Breast and cervical cancers account for about 50% of all cancers in Sudanese women. Routine screening for these cancers has markedly reduced the mortality in the developed world, but in developing countries, which largely lack screening programs, these two cancers remain the primary cause of death with combined crude mortality of about 18.5/100 000 (Hamad., 2006). In Sudan, the application of radioisotopes in early screening and post treatment prognosis was started in (RICK) and Sudan Atomic Energy Commission (SAEC) in 1980 including breast, colon, thyroid and other types of cancers. Many studies on tumor markers for early detection of cancers were performed in the country and particularly at (SAEC), including prostate, ovaries, breast and thyroid… etc. Analysis of hospital based data has been done for the evaluation of cancer status and incidence in Sudan. Ahmed *et al* 2009 reported that the problem of cancer in Sudan was increased twenty times since the first radiotherapy center was established.

To the best of my knowledge there were no previous studies on the late effects of scattering radiation in radiotherapy. The current study is the first study addressing the out of field scattering radiation and second cancer, hence this study was devoted to characterize the radiobiological effects of two different photon beam energies namely, 6 and 15MV used in radiotherapy. Chinese hamster V79 cell line was used in this study to measure the survival curve and the micronuclei induction, with special regards to the second primary cancer induction in long survivors after radiotherapy of deep seated tumors such as prostate cancer. Moreover this study was designed to study the characterization of different treatment modalities in radiotherapy and to measure the late effects of scattering radiation on survival curve and micronuclei as a biodosimetry tool, the results of this study may help the radiotherapists to give more attention to the scattering radiation, modulate the radiotherapy and offer more protection for their patients, and we have therefore used the micronuclei induction assay to characterize two photon beams with different energies 6 and 15 MV commonly used in radiotherapy, the photoneutrons dose for the 6 MV photon beam is zero as the photoneutrons reactions have higher energy thresholds (>8MV), whereas the 15 MV photon beam does produce low doses of photoneutrons which are difficult to be measured. These low neutrons doses are not cytotoxic but almost no data are available on their role in late effects induction, and how they may contribute to second primary cancer induction. The present work describes the results obtained using the rodent V79 cell line to measure the dose–effect curves for micronuclei and cytotoxicity induction after irradiation with photons at the two energies.

Objectives

General Objective:

The general objective of this study is to perform a radiobiological characterization, with special regard to late effects of different photon beam energies from linear accelerator used in radiotherapy and to compare different treatments modalities.

Specific objectives:

1. To determine the surviving fraction as a main radiobiological end point for two different photon beam energies 6 and 15 MV.

2. To study the late effects using micronucleus assay at scattering radiations for different treatment modalities.

3. To investigate the biological effect of photoneutron dose resulted from high energy photon beam (15 MV).

Chapter One

Literature Review

Although it is recognized that cancer etiology is complex and multifactorial, many causes of cancers have been investigated, one of the most important cause of cancer is radiation but still radiation can be used for cancer treatment. The use of radiation for treatment may lead to second primary cancer as a late effect of radiotherapy.

1.1 Types and sources of radiation:

Radiation is classified as ionizing and non-ionizing. Non-ionizing radiation ranges from extremely low frequency radiation, shown on the far left through the audible, microwave, and visible portions of the spectrum into the ultraviolet range. Non-ionizing radiation has very long wave lengths, and frequencies in the range of 100 Hertz or cycles per second. They carry low energies that are insufficient to eject orbital electrons from the atom. If the radiation has sufficient energy to eject one or more orbital electron from the atom or molecule the process is called ionization and that radiation is said to be ionizing radiation (Hall *et al*., 2006). Ionizing radiation includes electromagnetic radiation such as X-rays which originates from orbital electrons and Gamma rays which emitted as a nucleus undergoes transition from its higher excited state to lower excited state or ground state, while X-rays produced extranuclear when electrons are accelerated and stopped on a target (Sam, 2006). Another type of ionizing radiation is the particle radiation, which includes alpha particles these are positively charged helium nuclei, Protons and Neutrons and fission products. The energy of ionizing radiation is measured in kilo-electron volt (keV) Hall *et al* (2006). Natural sources of ionizing radiation include cosmic rays, gamma rays from the earth, radon decay products in air, and various radionuclides found naturally in food and drink. Artificially sources include medical X-rays, fallout from the testing of nuclear weapons in the atmosphere, discharges of radioactive waste from the nuclear industry (UNSCEAR 2000).

1.1.1 Units of radiation:

The objective of dosimetry and dose specification in radiobiology range from incidental estimates of radiation delivered to the precise specification of absorbed dose, its macroscopic and microscopic distributions and other quantities (Zoetelief and Broerse, 1998). Various radiation units and quantities are used to quantify the amount of radiation dose received. The exposure is the amount of charge librated when photons are completely stopped in air divided by the total mass of air. The unit of exposure is roentgen (R) which by convention is defined only for X and Gamma ray photons. Absorbed dose is defined as the energy absorbed per unit mass from any kind of ionizing radiation in any target. The unit of absorbed dose is gray (Gy). Gy is energy absorbed per one kilogram of matter (J/kg). Equivalent dose is the absorbed dose averaged over a tissue or organ and weighted for the radiation quality that is of interest. The weighting factor for this purpose is called "radiation weighting factor", which reflects the severity of biological effects due to different types and energies of radiation. When the incident radiation field consists of different types and energies of radiation, the equivalent dose in a tissue or organ is equal to the sum of the weighted absorbed doses and its unit is Sievert (Sv). Effective dose is the sum of the weighted equivalent doses in all the tissues and organs of the body. The factor by which the equivalent dose in tissue or organ is weighted is called "tissue weighting factor", which represents the relative contribution of that organ or tissue to the total detriment resulting from uniform irradiation of the whole body and its unit is Sievert (Sv).

Radiation field quantities are defined at any point in a radiation field. There are two classes of radiation field quantities referring either to the number of particles, such as fluence and fluence rate, or to the energy transported by them, such as energy fluence. The fluence, Φ is the quotient of dN by da, where dN is the number of particles incident upon a small sphere of cross-sectional area da, thus

$$\Phi = \frac{dN}{da}$$

The kerma, K, is the quotient of dEtr by dm, where dEtr is the sum of the kinetic energies of all charged particles liberated by uncharged particles in a mass dm of material. It is given by:

$$K = \frac{dEtr}{dm}$$

Absorbed dose, D, is defined as the quotient of *de*, by *dm*, where *de* is the mean energy imparted to matter of mass dm by ionising radiation, that is

$$D = \frac{d\varepsilon}{dm}$$

1.1.2 Interaction of photon with matter:

The probability of interaction of radiation with matter is dependence of on photon energy and chemical composition of the irradiated matter. At low photon energies, photo-electric effect where the incident photon delivered almost all of its energy to K or L shell electron, the liberated electron has a kinetic energy approximately equal to the energy of the photon minus the electron binding energy. The process of Compton scattering is predominates when an incident photon gives part of its energy to a free orbital electron and deflected from its original pass, Compton process dominates with high energy photons and more probable to occur in the outer shell electrons. Rayleigh or coherent scattering is an elastic scattering of the incident photon by the matter atom is neither ionized nor excited and the incident photon is deflected from its pass, for photon energies higher than 0.5 MeV the photon is scattered forward. Pair production is the process in which the annihilation of a photon in an electromagnetic field coincides with the creation of an electron-positron pair, and can only occurs when the energy of a photon is at least twice the rest energy of an electron i.e. 1.022 MeV. The probability of pair production starts at 1.022 MV and increased with increasing photon energy. In the case of the photonuclear absorption or photodisintegration a photon is absorbed by nucleus resulting in the ejection of one or more particles such as proton or neutrons. Due to the large binding energy of the particle in the nucleus a high threshold photon energy exists, below which the photonuclear absorption does not occur i.e. 8 MeV (Jansen., 1997).

1.1.3 Biological effects of ionizing radiation:

Whole body overexposure to ionizing radiation mainly occurs as a result of accidents with radiation sources, many of them have been documented by International Atomic Energy Agency, and it manifested by many symptoms include Vomiting, Diarrhea Headache, Body temperature, Lymphocytes, Granulocytes, Hair loss (González., 2007). All tissues are damaged by radiation. Tissues that undergo rapid renewal have earlier side effects that relate to depopulation i.e. Irradiation of the gastrointestinal tract causes

a series of biological effects that begin with immediate nausea and vomiting, delayed malnutrition and diarrhea, and which can progress to late fibrosis and necrosis. Slowly renewing, or non-proliferating tissues, have side effects that include inflammatory changes and devascularization such as the prominent fatigue syndrome associated with brain irradiation. The toxicity process is sequential and progressive in most tissues, but the pace varies between tissues and differs from patient to patient. Treatment doses are as much determined by the tolerance of the tissues being irradiated as they are by the tumor being treated (Okunieff *et* a*l*., 2008).

Quantitative cell survival curves provide the basis for the standard linear quadratic model used to interpret radiation effects. They have also provided insight into exceptions to the linear- quadratic model, including bystander effects, low-dose hyper-radiosensitivity (HRS), and induced radioresistance. Most cell survival data have been acquired using cultured cells and colony formation assays (Bladen 2007).

As early as the mid-1960s, ionizing radiation was known to be capable of inducing chromosome aberrations in the metaphase of human peripheral lymphocytes. Since then, the chromosome aberration assay has been widely used as a sensitive biomarker for dose reconstruction following radiation exposure. In particular, the analysis of dicentric chromosomes and rings (dicentric and ring chromosomes), two aberrations exemplifying inter- and intrachromosomal exchanges, respectively, has been generally considered to be the standard means for estimating biodosimetry based on its well-established dose–response relationship with radiation exposure and its low baseline levels in the general population (LIU *et* a*l*.,2009).

1.1.4 Interaction of radiation with biological system:

It is generally assumed that biological effects on the cell result from both direct and indirect action of ionizing radiation. Direct effects are produced by the initial action of the radiation itself and indirect effects are caused by the later chemical action of free radicals and other radiation products. The biological effects of radiation can differ widely depending on dose, kind of radiation, and observed end point. Irradiation of any biological system includes many steps firstly the physical phase which consists of interaction between radiation and the atoms of which the tissue is composed. A high-speed electron interacts with orbital electrons causing ionization or excitation.

Chemical interaction of these ionizations with biomolecules causing damages to DNA, free radicals produced by radiation in water are the creation of ionized and excited molecules, H_2O^+ and H_2O^*, and free subexcitation electrons. The biological effects include all the subsequent process examples acute effects and late effects (Steel 2002).

1.1.4.1 Ionizing Radiation and cancer:

Ionizing radiation is perhaps the most extensively studied human carcinogen. There have been a number of epidemiological studies on human populations exposed to radiation for medical or occupational reasons, as a result of protracted environmental exposures due to radiation accidents, or after atomic bombings. As a result of these studies exposure to ionizing radiation has been unambiguously linked to cancer causation (Sowa *et al*., 2006). Induction of cancers in humans by radiation was first documented by the appearance of cancers in scientists and radiologists exposed to radium salts and working with X-ray generators in the first few years after discovery of X-rays and radioactivity (Suit *et al*., 2007).

1.1.4.2 Stochastic effects:

Exposure to ionising radiation, even at low doses, may cause damage to the nuclear (genetic) material in cells that may result in the development of radiation induced cancer many years later, heritable disease in future generations and some developmental effects under certain conditions (ICRP, 2003a).

1.1.4.3 Tissue reactions (deterministic effects):

Tissue reactions occur at doses higher than the dose limits recommended in the protection system, and especially in accident situations, radiation exposures may cause deterministic effects (tissue reactions). These effects result from the impairment of the integrity and function of organs and tissues, clinically observable damage then occurs above a threshold dose. The extent of any damage depends upon the absorbed dose and dose rate as well as radiation quality. The expression of injury varies from one tissue or organ to another depending upon cellular radiosensitivity, the function of differentiated cells, cellular composition, and cell renewal capacity. Loss of reproductive capacity of cells, the development of fibrotic processes and cell death play a central role in the pathogenesis of most tissue reactions. Early tissue reactions include damage to

haematopoietic tissue, the cells lining the gastrointestinal tract, the basal cell layer in the skin, and the male germ cells. Late tissue reactions may also depend in part on damage to blood vessels or connective tissue elements that are essential for the functioning of all organs and tissues as well as of the lens of the eye. Such damage can be expressed many months or even years after radiation exposure (ICRP 2007b).

The weighting factors, W_T (later termed tissue weighting factors) accounted for the varying radiation sensitivity of tissues to the induction of stochastic effects. The wT values recommended by the Commission in Publication 26 were based on the risk of fatal cancer and of serious heritable disease in the first two generations (ICRP 2007b).

Table (1.1): Radiation weighting factors W_R as recommended by ICRP 2007:

Radiation type	**Radiation weighting factor W_R**
Photons	1
Electrons and muons	1
Protons and charged pions	2
Alpha particles, fission fragments, heavy ions, Neutrons	20

Table (1.2): Tissue weighting factor as recommended by ICRP 2007:

Organ/Tissue	**Number of tissues**	w_T	**Total contribution**
Lung, Stomach, Colon, Bone marrow, Breast, remainder	6	0.12	0.72
Gonads	1	0.08	0.08
Thyroid, Oesophagus, Bladder, Liver	4	0.04	0.16
Bone surface, Skin, Brain, Salivary gland	4	0.01	0.04

Causes of human cancers other than ionizing radiation will be highlighted in the following paragraphs.

1.2 Socioeconomic and dietary causes of cancer:

It is estimated that up to half of cancers diagnosed in the developed countries could be prevented if beneficial changes were made to a certain lifestyle and behavioral factors such as smoking, dietary habits, physical activity, alcohol consumption, and body weight (Li *et* a*l*., 2008). Primary prevention advices of the European Code against Cancer (ECC) were included in an educational intervention (EI) using social cognitive theories for motivating families with cancer experiences to adopt six cancer prevention behaviors reported that most prevalent tumors could potentially be prevented by educative interventions because they have been linked to sun, diet, and smoking (Jose *et* a*l*., 2007). In another study reported that women who start smoking at younger ages, and continue to smoke for at least 20 years increase their breast cancer risk, the study showed a consistent dose-response, which enhances the biological plausibility of an increased breast cancer risk due to smoking, and postulated that the plasma lipoproteins may transport the carcinogens in the tobacco smoke to the breast where they can be stored in breast adipose tissue and could then be metabolized and activated by human mammary epithelial cells. Furthermore, the breast tissue of smokers have a higher prevalence of smoking-specific DNA adducts and p53 gene mutations compared with that in nonsmokers. This all supports the likelihood of a positive association between cigarette smoking and breast cancer risk (Gram *et* a*l*., 2005).

Vegetables and fruits are associated with a reduced risk of cancers, especially lung cancer. The possible protective compounds in vegetables and fruits may include a wide variety of phytochemicals, colorful compounds that are abundant as pigments in plants, among them are the carotenoids, and the main carotenoids are α-carotene, β-carotene, lutein, zeaxanthin, β-cryptoxanthin, and lycopene. They are potent quenchers of free radicals, which are by-products of metabolic processes originating from environmental pollutants such as cigarette smoke (Voorrips *et* a*l*., 2000).

Calcium has the potential to reduce risk of colorectal cancer through a variety of plausible biological mechanisms, chief among these is the hypothesized ability of calcium to reduce the proliferative effect of secondary bile acids in the colon, secondary

bile acids produced during the digestion of fat, are highly irritating to the epithelial cells of the colon, but calcium forms insoluble soaps with these bile acids thus neutralizing their ability to irritate the epithelial surface of the colon and thereby induce an increase in proliferation rates. Without the increase in cellular proliferation rates, the likelihood of individual initiated cells progressing to neoplasia or cancer would presumably be diminished (Flood *et* a*l*., 2004).

1.3 Genetic susceptibility to cancer:

Most cancers are sporadic in other words, they are attributable to somatic mutations, however a proportion of cancers, which varies with the cancer type occur in individuals who are at a higher risk than the remainder of the population because of inherited predispositions due to specific germ-line mutations in one or another of the cancer genes (Sankaranarayanan and Chakraborty., 2001).

Over the last two decades, the genes responsible for familial cancer syndromes were cloned and identified using the positional cloning of candidate gene approach and an animal models of inherited tumor predisposition associated with tumor suppressor genes have also been discovered (Hino *et* a*l*., 2006). Approximately 50% of all humans inherit two deleted copies of the GSTM1 gene which encodes for the carcinogen detoxification enzyme glutathione *S*-transferase M1, these people have a high risk of bladder cancer, GSTM1 gene may modulate the internal dose of environmental carcinogens and thereby affect the risk of developing bladder cancer and absence of the GSTM1 gene encoding the glutathione *S*-transferase M1enzyme significantly increased risk to persons with exposure to carcinogens. It is estimated that 25% of all bladder cancer may be attributable to the at-risk GSTM1 0/0 genotype (Douglas *et* a*l*., 1993).

1.4 Viral infections and cancer:

Infections have been recently identified as important etiologic factors for an increasing percentage of human cancers. Between15 to 20% of the global burden of human cancers have been linked to viral, bacterial and parasitic infections (Hausen., 2001). Viruses must be thought of as the second most important risk factor for cancer development in humans, exceeded only by tobacco consumption (Hausen., 1991). Oncogenes encoded by human tumor viruses play integral roles in the viral conquest of the host cell by

subverting crucial and relatively non-redundant regulatory circuits that regulate cellular proliferation, differentiation, apoptosis and life span, and may disrupt pathways that are necessary for the maintenance of the integrity of host cellular genome. Some viral oncoproteins act as powerful mutator genes and their expression dramatically increases the incidence of host cell mutations with every round of cell division. Others subvert cellular safeguard mechanisms intended to eliminate cells that have acquired abnormalities that interfere with normal cell division. Viruses that encode such activities can contribute to initiation as well as progression of human cancers (Münger *et* a*l*., 2006). Presently between 15 to 20% of the global cancer incidence can be etiologically linked to specific infections. Bacteria (Helicobacter pylori) and helminthes (Schistosoma, Opistorchis, and Clonorchis) contribute to the development of bladder and rectal cancers and to cholangiocarcinomas. Viruses however are the main cancer risk factors. Hepatitis B and C virus infections are involved in hepatocellular carcinomas. Specific types of papillomaviruses cause one major human cancer, cancer of the cervix and play an additional role in a number of other anogenital, but also in some oropharyngeal and cutaneous cancers. Epstein–Barr virus, known as a human tumorvirus since 1964, human herpes-virus types 8 (HHV-8), and human T-lymphotropic retrovirus type 1 (HTLV-1) have also been identified as human tumor-viruses (Hausen., 2001).

1.5 Other carcinogens:

In addition to the above mentioned cancer risk factors there are some factors arise from the occupational exposure to carcinogens, as radiation effect has been addressed previously, in this paragraph other carcinogens will be discussed. . Many recognized human carcinogens are occupational carcinogens. There is a large volume of epidemiologic and experimental data concerning cancer risks in different work environments (Siemiatycki *et* a*l*., 2004). The International Agency for Research on Cancer (IARC 2002) has classified 150 chemical or biological agents as known or probable human carcinogens, and exposures to many of these carcinogens for example asbestos, cadmium and benzene occur in occupational settings. Occupational exposure is defined as any contact between the human body and a potentially harmful agent or environment in the workplace (Driscoll *et* a*l*., 2004**)**. Exposures to occupational carcinogens, such as asbestos, are also well-established causal factors of lung cancer;

asbestos refers to a group of naturally occurring fibrous silicate minerals, which have been widely exploited in the building industry (Kettunen *et* a*l*., 2009). Cytogenetic damage in individuals occupationally exposed to pesticides has received the attention of investigators in several countries. A high rate of MN and DNA damage in pesticide-exposed individuals in addition, some effects of genetic polymorphisms in paraoxonase (PON) in the modulation of MN results were observed in the exposed group, and an association between *GSTM1*, *GSTT1* and *CYP2E1* polymorphisms was suggested (Silva *et* a*l*., 2008).

1.6 Treatment of cancers:

Different modalities are used for cancer treatment, either single regimen or combination therapy, which is usually used for more effective treatment and to avoid unwanted effects. Radiation therapy is an important local cytotoxic modality for cancer treatment whose aim is to control the disease while minimizing damage to normal tissue. The combination of different treatment modalities offers a more effective cure and reduction in normal tissue toxicity (Rosenthal., 1996).

1.6.1 Surgery:

Surgeons who perform cancer surgery work closely with their pathologists. The preoperative planning requires accurate assessment of tissue diagnosis and resected specimens are sent for final histopathological diagnosis, tumor grading, margin assessment, and clinicopathological staging. Expression of specific biomarkers in the resected primary tumor is also assessed. Breast surgeons are familiar with receptors expression in breast cancer, urologists follow prostate-specific antigen tests and gastrointestinal surgeons order carcinoembryonic antigen tests for their colorectal cancer (David and Nelson, 2008). Complete surgical excision is the main treatment, and is generally all that is required for some types of tumors for example, dermatofibrosarcoma protuberans (DFSP). Mohs-surgery offers the advantage of precise and complete evaluation of the entire peripheral and deep margin, resulting in extremely low reported local recurrence rates (Sondak *et* a*l*., 1999). Axillary node dissection is a component of modified radical mastectomy, and also is commonly used in breast conserving surgery, it physically removes potentially cancerous tissue in the

axilla and allows for adequate staging information as a guide to more appropriate therapy (Du X *et* a*l*., 2002).

1.6.2 Chemotherapy:

Cytotoxic agents form the basis of most cancer chemotherapies. These agents primarily affect rapidly proliferating cells, so their uses incur morbidity associated with damage to tissues, such as bone marrow and gastrointestinal mucosa. Clinical outcome would be improved if it were possible to develop therapeutics with more specific activity against p53-deficient cancers, which account for over 50% of all cases (Dixon, 2002).

1.6.3 Radiotherapy:

External beam radiotherapy (EBRT) is a treatment option for patients with early-stage or locally advanced cancer. Recent data suggest that local control of cancer is directly related to the radiation dose which would in turn result in improved biochemical control, and increased overall survival. Various dose escalation methods are available including three-dimensional conformal radiotherapy (3D-CRT) or intensity-modulated radiotherapy (IMRT) (Joseph *et* a*l*., 2008). Postoperative radiation therapy substantially decreases local relapse and moderately reduces cancer mortality, but can be associated with increased late mortality due to secondary malignancies (Weber *et* a*l*., 2006). Accuracy of the delivered dose to the patient is one of the most important and effective factors in radiotherapy (Mesbahi *et* a*l*., 2007).

1.6.3.1 Radiotherapy related parameters:

D¨orr and Herrmann in 2002 identified the radiotherapy-related parameters influencing the development of second malignancies in a population of about 31 000 patients who were treated with radiotherapy in Dresden, between 1969 and 1989, and found that of these patients, 203 were re-admitted for radiotherapy of a newly developed malignancy. Based on completeness of documentation, definition of site of origin and exclusion of recurrent or metastatic tumours, 85 patients were selected for analysis of spatial relation between new tumour and primary treatment fields, and of dose at the site of origin. He reported that about 50% of new tumours were observed in the margin region of the initial treatment volume, while approximately 10% were seen within the field, and a

comparable number (>10%) in the adjacent region and about 30% in the distant region. Regarding the dose delivered in radiotherapy about 58% of all tumours developed within a volume that received a dose <6 Gy at first treatment. In contrast, about 35% of the tumours developed at doses between 10 and 30 Gy, and a few tumours developed at higher doses. Therefore, a clear increase in tumour frequency is observed within the low dose volumes, the increased incidence in this region suggests that radiation may at least contribute to tumorigenesis, which may or may not be accompanied by genetic susceptibility or other factors of the patient. Similar results were obtained in a group of 31 patients initially irradiated for benign diseases. This should be considered in radiotherapy, particularly with multiple-field techniques or intensity-modulated radiotherapy, where the volume receiving <6 Gy is substantially increased (D¨orr and Herrmann, 2002).

1.6.3.2 Scattering radiation in ^{60}Co unit versus LINAC:

Dosimetry quality audits and intercomparisons in radiotherapy centers is a useful tool in order to enhance the confidence for an accurate therapy and to explore and dissolve discrepancies in dose delivery. It is reported that ^{60}Co units had worse performance mechanical characteristics than LINAC they concluded that dosimetry accuracy was much worse in ^{60}Co units than in LINAC. 61% of the ^{60}Co units exhibited deviations outside the treatment field, organs at risk (OAR) doses were slightly higher for the ^{60}Co plans than for LINAC and planning target volume (PTV) coverage for the LINAC was better than that for the ^{60}Co plans (Hourdakis and Boziari., 2008). The need for an increase in the number of fields compared with the 10MV plan, to maintain normal tissue doses, made ^{60}Co plans less efficient than the corresponding LINAC plan. ^{60}Co has long been cited as being generally unsuitable for deep seated tumor treatment modality (Adams and Warrington., 2008).

The primary advantage of accelerator over ^{60}Co has been their higher dose rate and more uniform dose. Patients thus need shorter treatment and the length of time it takes to provide the daily treatment does not vary. High megavoltage LINAC permits treatment that is impossible with ^{60}Co. A combination of greater depth dose and deeper build up, maximum dose is delivered far below skin surface and healthy tissues receive far less dose. Malignancies such as cancers of the cervix, prostrate and urinary bladder are best treated with high energy machines. Additionally, high energy beams permit

simple treatment planning from the anatomical point of view. An added advantage of the LINAC is that it gives electron beams of various energies. These electron beams offer the advantages of rapid and sharp fall off in depth dose, variable depth of penetration, less bone absorption than x-ray beams, and decreased radiation build up. The superficial tumours are best treated with electron beams. ^{60}Co has 1.25 MV average photon energy and half life of 5 years and 0.5 cm skin sparing effect. Initially stationery units were in practices which were followed by rotational units centering around 60 cm, 80 cm even 100 cm (ISO Centre). Linear accelerator has electron energies 5, 6, 7, 8, 10 and 12 MV and field besides photon energies of more than10 MV (Koul *et* a*l*., 2001).

1.6.4 Out-of field secondary radiation and neutrons:

A consequence of any form of radiation therapy is secondary radiation outside the treatment field that affects healthy tissues and potentially sensitive organs, the role of out-of field secondary radiation during using of high energy linear accelerator in treatment of deep seated tumors such as carcinoma of the uterine cervix or carcinoma of prostate, patients may receive a large total body dose of scattered X-rays, depending in part on accelerator design, shielding and treatment geometry and also a small but significant dose of photoneutrons (Hall *et* a*l*.,1995). Neutrons are produced primarily through the interaction of photons with energy more than 8MV with high Z materials such as tungsten or lead in the linear accelerator head. Neutrons are of particular importance because of their high relative biological effectiveness (RBE). Neutrons encountered around medical accelerators have a radiation weighting factor of 20 meaning that neutrons are approximately 20 times as biologically damaging as photons. Near medical accelerators the out-of field photon dose is typically larger than the out-of field neutron dose but because of their large weighting factor neutrons may be the dominant source of tissue damage from secondary radiation (Stephen *et* a*l*., 2005).

It should be expected; therefore patients treated with high energy linear accelerators might show a higher incidence of second malignancies, the number of additional malignancy that might result from the use of high energy linear accelerators and the inevitable presence of photoneutrons taking into account the photoneutrons dose and cancer risk available from the literature reported by the ICRP 91 (Hall *et* a*l*., 1995).

1.6.5 The impact of 3D-CRT and IMRT in radiotherapy:

The latent time to clinical manifestation of second primary tumours for low-energy x-rays was reported to be about 18 years. Gamma-irradiation was applied preferentially in the period after 1985. Therefore, more second primary tumours might be expected in further follow-up investigations. It has been taken into consideration for modern multi-field radiotherapy plans, as well as for intensity-modulated radiotherapy (IMRT), that the low-dose volume is significantly increased compared with opposing-field techniques. However, low-voltage x-ray or telecobalt beams are associated with a substantially smaller fall-off in dose, and hence a larger low-dose volume, at the beam margins than a linear accelerator beam (D¨orr and Herrmann., 2002).

The transition from conventional radiotherapy to three-dimensional conformal radiation therapy (3D-CRT) involve a reduction in the volume of normal tissues receiving a high dose, with an increase in dose to the target volume that include the tumor and a limited amount of normal tissues. One might expect a decrease in the number of carcinomas all around (Hall *et* a*l*., 2003). Many of the most important advances in radiation therapy have resulted from innovation in technology and engineering, for example the introduction of megavolt machines, such as cobalt units and linear accelerators, both spinoffs from world war Π technology, followed by the computer revolution applied to treatment planning these techniques have culminated in the sophisticated technique of intensity-modulated radiation therapy (IMRT), which allows dose to be concentrated in the tumor volume while sparing normal tissues. This property is a major step especially for children, in whom sparing normal tissues to avoid subsequent growth detriment is critically important; however the downside to IMRT is the potential to increase the number of radiation induced second cancers (Hall *et* a*l*., 2006).

When IMRT is considered as a replacement for conventional treatment, two factors must be taken into account, firstly more monitor units are used, which results in larger total-body radiation dose and secondly, more fields are used which results in a larger volume of normal tissue exposed to lower radiation doses. Compared with 3 dimensional conformal radiation therapy (3D-CRT), IMRT may double the incidence of solid cancers in long-term survivors because of combination of the increase in monitor units and the changed dose distribution (Hall *et* a*l*., 2006).

1.6.6 Second primary cancer after radiotherapy:

At the present time, approximately 10% of patients who present at major cancer centers have a second primary malignancy causes may be related to treatment of previous malignancy. In radiotherapy patients the induced tumor includes carcinomas that may appear in sites adjacent to or remote from the treated area the number of carcinoma is relatively large but the relative risk is small. In addition sarcomas may appear in heavily irradiated tissues, either within or close by the treatment field. In radiotherapy patients sarcomas are small in numbers but are characterized by a large relative risk. The prime sites for developing a second malignancy are colon, lung and stomach characterized by high probabilities Radiation induced tumor in radiotherapy patients will become increasingly important as younger patients are treated and improved cure rates obtained (Hall *et* al., 2006).

The incidences of second malignancy by conventional radiotherapy include incidence of carcinomas in tissues such as the gastrointestinal tract, breast, thyroid and bladder, which is linear with dose up to about 2.5 Gy. There is great uncertainty concerning the higher dose response relationship, some data suggest a decrease at higher dose, usually attributed to cell killing. Radiotherapy patients also show an incidence of carcinomas, often in sites remote from the treatment field and sarcomas in heavily irradiated in field tissues. Radiotherapy has been shown to be associated with a statistically significant, though very small enhancement in the risk of second malignancies, particularly in long term survivors. The incidence of radiation-induced second tumors can be assessed by comparing patients receiving radiation therapy with those for whom surgery was the initial treatment chosen. Another study on data from the National Cancer Institute's Surveillance Epidemiology and End results (SEER) program concluded that the risk of second solid tumor of any type and at any time post diagnosis was significantly greater after radiotherapy than after surgery, by about 6%. The increase relative risk (RR) became greater with time and reached 34%after 10 years or more (Relative Risk is an epidemiologic term in which the risk of a disease resulting from radiation is expressed as some percentage increase of the normal rate of occurrence of the disease). The most dramatic increases were for the bladder (77%) and the rectum (105%) for 10 years or more after diagnosis. For sarcomas produced in the heavily irradiated tissues within the field, RR of 145% compared with surgical patients was observed at 5 years and longer

time. In radiation therapy for the carcinomas of the cervix at very high doses, on the order of several hundred Grays, there is an increase in the risk of cancer of the bladder, rectum, vagina, possibly bone, uterine corpus and cecum and of non-hodking's lymphomas, where doses of several Grays increase the risk of stomach cancer and leukemia (Hall and Wuu, 2003).

The increased incidence of breast cancer in women after previous radiotherapy for Hodgkin's disease is well described and is attributed to the incidental inclusion of portions of the breast in the portals used to irradiate the mediastinum with or without Infraclavicular/axillary regions. Radiation induced fibrosarcoma and osteosarcoma as a side effect of therapeutic irradiation for breast carcinoma were reported (Deutsch *et* a*l*., 2001).

1.6.6.1 Secondary Malignant Neoplasm's (SMNs) in children:

Childhood cancer survivors are at 19-fold more increased risk for developing another malignancy, The Childhood Cancer Survivors Study (CCSS) reported 314 second malignant Neoplasm's (SMNs) in 13 518 childhood patients treated from 1970 to 1986 united state, which yields a standardized incidence ratio (SIR) of 6.38, with the largest excess observed for bone cancer (SIR: 19.14) and BC (SIR: 16.18). An increased risk for SMNs was seen in females and those who were at a younger age when diagnosed. Twenty years after cancer diagnosis, the cumulative estimated SMN incidence was 3.2% (Dickerman, 2007). One of the most alarming long-term consequences of childhood cancer is the occurrence of a second primary malignancy. Treatment-related cancers are a well-recognized sequel of radiotherapy and an excess risk of subsequent malignancies of the thyroid, breast, bone, soft tissue, and central nervous system (CNS) following radiation treatment for childhood cancer have been reported (Ron., 2006). An increased susceptibility of children to radiation-induced cancer is biologically plausible because of the fact that their tissues are still growing and therefore the dividing cells are more prone to somatic genetic damage. In addition, children have a longer life expectancy during which oncogenic effects may develop (Sadetzki and Mandelzweig., 2009). A number of Treated childhoods Hodgkin disease patients have developed late effects, such as secondary malignancies. These cancers may occur years to decades after remission and arise in the breast, thyroid, gastrointestinal tract, lung, skin, urogenital tract, and brain. There is also an increased risk of leukemia and non-Hodgkin

lymphoma, etiology and risk factors for each cancer type vary but often include certain radiation dosages (Lin and Teitell., 2005).

Use of IMRT for children treatment represents special case for three reasons, firstly children are more sensitive to radiation induced cancer than are adults by a factor at least 10, second radiation scattered from the treatment volume is more significant in the small body of the child than in the larger body of an adult, third is the question of genetic susceptibility. Many of the cases of childhood cancers involve a germ line mutation that may confer susceptibility to radiation induced cancer. Few things are worse for patients than to survive the initial treatment, live with the long term morbidity of therapy, only to find that they have developed a radiation-induced second cancer with a worse prognosis than the original tumor (Hall *et* a*l*., 2006).

1.6.7 Stereotactic Radiosurgery:

Stereotactic radiosurgery was first described by Leksell in 1951 as "Delivery of a single high dose of radiation to a small and critically located intracranial volume through the intact skull.'' Stereotactic radiosurgery allows non-invasive cerebral surgery to be performed with greater precision while minimizing risk to the surrounding tissues (Viola *et* a*l*., 2006).

1.6.8 Brachytherapy:

Radiation can be given directly into the tumor tissue by the implantation of radio-isotopes, a procedure referred to as brachytherapy (Denis 2000). (HUYGHE *et* a*l*., 2009) concluded that most men treated with brachytherapy have conserved ejaculatory function after prostate brachytherapy.

1.6.9 Combination therapy:

Combined-modality treatment using chemotherapy and radiotherapy, particularly concurrently, has become the standard of care for the treatment of many solid tumors due to documented significant improvements in locoregional disease control and overall survival. The chemotherapeutic agent 5-fluorouracil (5-FU) is the most commonly used radiosensitizer (Adamsen *et* a*l*., 2009). Future improvements in the therapeutic index for radiotherapy depend on increasing the sensitivity of tumor cells to radiation and

reducing the effects of radiation on normal tissues. Several agents and drug combinations including carboplatin, cisplatin, 5-fluorouracil, ifosfamide, etoposide, and most recently taxanes have been used as radiation sensitizers (Javvadi *et* a*l*., 2008).

1.7 Genotoxicity of ionizing radiation:

The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis. The observation that chromosome damage can be caused by exposure to ionizing radiation or carcinogenic chemicals was among the first reliable evidence that physical and chemical agents can cause major alterations to the genetic material of eukaryotic cells. Although our understanding of chromosome structure is incomplete, evidence suggests that chromosome abnormalities are a direct consequence and manifestation of damage at the DNA level for example, chromosome breaks may result from unrepaired double strand breaks in DNA and chromosome rearrangements may result from misrepair of strand breaks in DNA. It is also recognized that chromosome loss and malsegregation of chromosomes (non disjunction) are an important event in cancer and ageing and that they are probably caused by defects in the spindle, centromere or as a consequence of under condensation of chromosome structure before metaphase. In the classical cytogenetic techniques, chromosomes are studied directly by observing and counting aberrations in metaphases. This approach provides the most detailed analysis, but the complexity and laboriousness of enumerating aberrations in metaphase and the confounding effect of art factual loss of chromosomes from metaphase preparations has stimulated the development of a simpler system of measuring chromosome damage (Fenech., 2000).

1.8 Cytogenetic methods:

Cytogenetic biological dosimetry can make valuable contributions to the medical management of patients in the early period after a radiation disaster, where a rapid confirmation of dose is required. At such time, all that is needed is a rapid confirmation of clinical triage, based on approximate dose estimation using biological and clinical endpoints to a selected cohort, rather than precise dose estimations for a vast number of individuals. Radiation exposure induces many types of chromosomal aberrations in the exposed individual's peripheral blood lymphocytes (Prasanna *et* a*l*., 2005).

1.8.1 Dicentric assay:

In this assay, peripheral lymphocytes were separated from the blood and stimulated to divide in culture. Then using standard staining or hybridization with centromere-specific flouro in situ hybridization (FISH) probes, metaphase chromosome spreads were scored for the observed frequency of chromosomes that have two centromeres, the so-called dicentric chromosomes. Radiation dose is then estimated from comparison to a standardized curve obtained from human lymphocytes irradiated in vitro. While significant increases in dicentric frequencies have been documented following in vitro doses above 0.02 Gy, practical detection limits for in vivo exposures appear to be closer to 0.5 Gy (Loats *et* a*l*., 2007).

1.8.2 Comet assay:

The single-cell gel electrophoresis (SCGE) or comet assay was used to measure the amount of DNA fragmentation in individual cell. In this assay, a cell with fragmented DNA has the appearance of comet with brightly fluorescent tail the intensity of which represents the relative amount of DNA strand breaks present (Duty *et* a*l*., 2002).

1.8.3 Micronucleus assay:

Another cytogenetic assay used for biodosimetry was the scoring of micronucleus formation. This method has several advantages over the dicentric assay in that it requires less specialized expertise, more rapid and hence can more readily be applied to monitoring large populations. In this assay, lymphocytes were mitogenically stimulated in culture, then cytokinesis was blocked by cytochalasin-B. This results in mitosis and nuclear division without cell division. Extranuclear chromatin particles (micronuclei) were then counted in binucleated cells. Unlike the fairly radiation-specific dicentric assay, micronuclei can be induced by a range of other clastogens, including cigarette smoking and exposure to clastogenic chemicals. While most spontaneously arising micronuclei contain centromeres, it has been found that the majority of radiation induced micronuclei represent acentric fragments formed by chromosome breakage (Amundson *et* a*l*., 2007).

Micronuclei (MN) were small extranuclear bodies that arise in dividing cells from acentric chromosome/chromatid fragments or whole chromosomes/chromatids that lag behind in anaphase and were not included in the daughter nuclei in telophase. The cytokinesis-block micronucleus (CBMN) assay was the most extensively used method for measuring MN in human lymphocytes, and can be considered as a cytome assay covering cell proliferation, cell death and chromosomal changes. The key advantages of the CBMN assay lie in its ability to detect both clastogenic and aneugenic events and to identify cells which divided once in culture (Mateuca *et* a*l*., 2006). The micronucleus assay in lymphocytes was one of the most valuable tools in biological dosimetry. This was primarily due to the comparatively simple scoring procedure which allows the screening of many individuals within a short time interval assisted by automatic systems. There is however, an observation that seems to limit the usefulness of the assay in the high dose range after low LET radiation, the dose response curve start to level off at about 5–7 Gy and after high LET radiation at about 3–4 Gy. This phenomenon was well known also for other cytogenetic endpoints, such as dicentrics, and was interpreted as selection against heavily damaged cells. That means those cells with the highest amount of damage cannot enter mitosis so that neither dicentrics nor micronuclei can be expressed. Consequently, it was not possible to come up with reliable dose estimates for doses exceeding about 7Gy of low LET radiation, when one looks at micronucleus frequencies. Even worse, starting at about 10 Gy, one observes a reduction in the number of micronuclei, so that it was not possible to discriminate, for example, a dose of 7 and 15 Gy, when one looks at micronucleus frequencies only in binucleated lymphocytes (Müller and Rode., 2006).

(Bonassi *et* a*l*., 2007) reported that the presence of an association between MN induction and cancer development was supported by a number of observations. The most substantiated include the high frequency of MN as biomarker in untreated cancer patients and in subjects affected by cancer-prone congenital diseases, e.g. Bloom syndrome or ataxia telangiectasia, the presence of elevated MN frequency in oral mucosa, used as a surrogate biomarker of cancer in clinical chemoprevention trials, the correlation existing between genotoxic-MN inducing agents and carcinogenicity, e.g. ionizing and ultraviolet radiation and the inverse correlation between MN frequency and the blood concentration and/or dietary intake of certain micronutrients associated with reduced cancer risk, such as folate, calcium, vitamin E and nicotinic acid. Further

evidence based on the mechanistic and experimental correlation existing between chromosomal aberrations (CA) and MN which in most cases demonstrated that the frequency of CA in PBL of healthy subjects was a predictor of cancer risk. Recently the results of a long term international collaborative study on the possible association of lymphocyte MN frequency with cancer risk have been published. The conclusions of this study, performed on about 6700 subjects from 10 countries, provided preliminary evidence that MN in PBL was a predictive biomarker of cancer risk within a population of healthy subjects.

1.9 Cell Survival Assay:

The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone, this cell was then said to be clonogenic. A cell survival curve was therefore defined as a relationship between the dose of the agent used to produce an insult and the fraction of cells retaining their ability to reproduce. Although clonogenic cell survival assays were initially described for studying the effects of radiation on cells and have played an essential role in radiobiology, they are now widely used to examine the effects of agents with potential applications in the clinic. These include, in addition to ionizing radiation, chemotherapy agents such as etoposide and cisplatin, antiangiogenic agents such as endostatin and angiostatin, and cytokines and their receptors, either alone or in combination therapy. Survival curves have been generated for many established cell lines growing in culture. One can use cell lines from various origins including humans and rodents. These cells can be neoplastic or normal (Munshi *et* a*l*., 2005).

Chapter two

Materials and Methods

In this study the radiobiological effect of two different photon beams (6 and 15MV) were evaluated using the biological system; Chinese hamster V79 cell line to measure the survival curve and micronuclei induction. V79 lung fibroblasts cell line was established from Chinese hamster lung cells (Simi *et* a*l*., 1999) and is being widely used for somatic cell genetics and mutagenesis studies particularly advantageous for many practical studies for this cell. The doubling time is slightly more than one week since these cells grow rapidly in culture, the cycle time in mono layer is about 12 hours large number of colonies can be produced quite economically both in term of time and media costs. Eagle's minimal essential medium (MEM) supplemented with foetal calf serum, glutamine, gentamycine and HEPES and incubated at 37ºC in humidified 2% CO_2 atmosphere. Cells were exposed to dose range of 0.25, 0.5, 1.0, 2.0, 4.0 and 7.0 Gy using linear accelerator with energy of 6MV and 15MV respectively and recultured stained and scored for survival curve and micronucleus induction.

2.1 Reagents:

Chinese hamster V79 Cell line was obtained from Napoli university and the following reagents were obtained by Sigma-Aldrich Corporation located in Milan. Italy, and were used in this study:

- Eagle's Minimal Essential Medium (MEM. M3786).
- Foetal calf serum (MF00132239).
- Glutamine (MFCD0008044).
- Gentamicin (MFCD00270181).
- HEPES buffer (MFCD00006158).
- Trypsin (MFCD00082094).
- Phosphate Buffer Saline (MFCD00131855).
- Colcemid (MFCD00075459).
- Canada balsam (MFCD132800).
- Acetic acid (MFCD00036152).

- Ethanol (MFCD 459836).
- Methanol (MFCD 179337).
- GEIMSA stain (MFCD 00081642).

2.2 Equipment:

High energy X- rays (6 and 15 MV) used in this study were produced by a therapy level linear accelerator type LINAC-VARIAN DHX MLC (Varian Medical Systems. Italia, S.p.A. Cernusco). The LINAC is located at ISTITUTO NAZIONALE DEI TUMORI in Milano Italy. In addition the following equipment were used in this study :

- CO_2 incubator Premium U9270-0002
- Laminar flow hood (304 Stainless Steel Astec).
- Pipettes (Eppendorf 022470353 pipette 100-1000).
- Tissue culture flasks (18V4970 VARDS National Science).
- Pipette controller (Brand Tech accu-jet pro-No 154926).
- Parafilm "M" roll size 4 in × 125 ft (SIGMA G4507).
- Zeiss inverted microscope (Carl Zeiss Id03 Serial # 475983)
- Centrifuge 15V2341
- Colony counter (CAT. NO. MT2436).
- Coulter cell counter (Multisizer T3 COULTER COUNTER 6605697).

2.3 Cell line adaptation:

V79 cell-line was obtained from Napoli university laboratory and incubated in Degli university department of medical physics laboratory for adaptation and proliferation check for two days in their same medium. After two days incubation, cells were removed from the medium, washed in 5ml phosphate buffer saline (PBS) and trypsinized in 1ml trypsin, incubated for 5-7 minutes and 12 ml of their same medium added and heavy suspended, then divided into 6 new T-75 cm^2 flasks with 30ml of different new media, two flasks containing same Napoli medium and two flasks with Alfa-medium and the last two flasks were with MEM-medium. Each medium contains 10% Foetal calf serum (FCS), 2mM glutamine, 50ug/L gentamycine and 10mM HEPES Buffer., the 6flasks incubated at 37ºC in humidified 2%CO_2 and 95% air and checked after 1 day and their proliferation was very nice.

2.3.2 Trypsinisation and Counting of cells:

The 6 large flasks were trypsinized by removal of medium, washed by 5ml phosphate buffer saline, 2 ml trypsin solution was added and incubated for 5-7 minutes at 37ºC in humidified 2%CO_2 atmosphere, hence a suspension solution was obtained by adding 8ml of medium and shaking of each flask. Then from this suspension 0.5ml was taken in 19.5ml of isotonic solution, mixed and counted twice in Coulter's counter.

2.3.3 Maintaining healthy culture:

V79 cells were routinely cultured in Eagle's Minimal Essential Medium (MEM) supplemented with 10% foetal calf serum (Gibco, Grand Island, NY), 2mM glutamine, 50ug/L gentamycine (Gibco) and 10mM HEPES buffer. V79 cell cultures were maintained in exponential growth at 37ºC in humidified 2%CO2 and 95% air by daily watching and diluting the medium containing flask according to Prise *et al* method (Prise *et* a*l*., 2000).

2.3.4 Plating Efficiency (PE):

Plating efficiency of cell line indicates the percentage of cells seeded that grow into colonies. In this experiment, suitable number of cells was gently mixed with a medium and incubated for one week. Then the colonies were fixed directly by removal of the medium, washed in PBS, fixed in 70% ethanol, incubated for 7minutes then ethanol was eliminated and samples were dried at room temperature for one day. Then all samples were stained by 10% GIEMSA stain and allowed to stand for 20 minutes and then samples were washed three times with tap water, dried overnight and counted under colony counter according to Hall's method (Hall *et* a*l*., 2006) and the plating efficiency was calculated according to the following formula:

$$\boldsymbol{Plating\ efficiency(PE)} = \frac{\boldsymbol{Colonies\ counted}}{\boldsymbol{Cells\ seeded}} \times \mathbf{100}\%$$

2.4 Protocol for karyotype analysis:

The procedure of Hanigan and Pilot as modified by Xu and his group was used for karyotype analysis assay. V79cells were seeded into two T-25 flasks with ($2x10^5$) cells/flask and complete medium, maintained in exponential growth at 37ºC with 2% CO_2 and 95% air. After 24 hours colcemid was added at final concentration of (0.1µg/ml) and samples were incubated at 37ºC, after 3 hours medium was removed and cells were harvested by trypsinization and resuspended in new medium, centrifuged for 10 minutes at 2000 r/ minutes then medium was removed and cells were detached by shaking and rinsed in PBS solution and centrifuged at 2000 r/ minutes for 10 minutes and then PBS was removed. Hypotonic solution 0.075 m KCl was added and samples were incubated at 37ºC. After 20 minutes samples were centrifuged for 5 minutes at 800 r/ minutes. Samples were fixed using acetic acid: methanol- 1:3 and allowed to stand for 20 minutes at room temperature and centrifuged at 1000 r/ minutes for 5 minutes. Fixation step was repeated then fixative was removed and 2 drops of fresh fixative were added, samples were resuspended by gentle shaking and spreaded on slide and dried at room temperature for 1 day. Samples were stained with10% solution GIEMSA and left at room temperature for 15 to 20 minutes. Slides were covered with covering glass and stay for 1day at room temperature. Samples were scored under light microscope using magnification 100 and SEIGMA comertial-oil (Sargent *et* a*l*., 1996*)*. This protocol was used in this study with some minor modifications which could be summarized as follows; previously cultured cells were trypsinized, counted and reseeded in two T-25 cm^2 flasks with final cell numbers of ($2x10^5$) cells/flask and 6ml complete MEM Medium, incubated for 24 hours at 37ºC in humidified 5%CO2 atmosphere and 95% air. After 24 hours incubation colcemid was added at final concentration of 0.1µg/ml, and then samples were incubated for metaphase chromosome samples were incubated at 37ºC for 3 hours. After 3 hours later medium was removed and cells were trypsinized as mentioned above. New medium was added and cells were centrifuged for 10 minutes at 2000 r/ minutes at room temperature. Medium was removed and belt cells were detached by gentle shaking. Then PBS solution was added and samples were centrifuged for 10 minutes at 2000 r/min at room temperature. Hypotonic solution 0.075 m KCl was added and cells were incubated for 20 minutes at 37ºC, again cells were centrifuged for 5 minutes at 800 r/ minutes at room temperature. Fixative (acetic acid: methanol- 1:3) was added and allowed to stand for 20mins at room

temperature. Samples were centrifuged at 1000 r/mn for 5min. The above fixation step was repeated and fixative was removed, then 1or 2 drops of fixative were added and the cells pellet was resuspended by shaking. Then 1-3 drops of the cells suspension were spreaded on slide and left to dry at room temperature for one day. After one day slides were stained with10% GIEMSA solution and left at room temperature for 15 to20 minute. Finally slides were covered with covering glass and stay for 1day at room temperature. The magnification 100× microscope was used for chromosomal counting.

2.5 Irradiation of cells:

For the main experiment, previously cultured cells were irradiated with full medium in a water phantom at room temperature. The doses were 0.25, 0.5, 1.0, 2.0, 4.0, and 7.0 Gy for both 6 MV and 15 MV photon beams. Samples were irradiated in a water phantom in a field of 20X20 cm^2 and at a depth of 5cm in the isocenter position and SSD 100 cm and the suitable monitor units were calculated for each dose by the working technicain. Irradiation was performed at ISTITUTO NAZIONALE DEI TUMORI in Milano using the linear accelerator LINAC-VARIAN DHX MLC. This part of the experiment was done with the assistance of the working technician team using ionizing chamber IONEX DOSIMETER 2590 and FARMER NE 2581 according to the routinely used procedure for patients' treatment.

2.6 Cell Survival assay:

For survival or colony forming assay samples from the above suspension was also plated into T 25-flasks (five flasks for each dose) with cells at appropriate numbers with 6ml complete medium in each flask five flasks were used for each dose incubated for 6 days at 37ºC. after this period medium was removed and samples washed with PBS and fixed with 70% ethanol (for10 minutes), dried for two hours and stained with 10% GIEMSA solution for 20 minutes dried overnight and counted under colony counter. For evaluation of survival fractions, colonies with more than 50 cells were scored as survived. Plating efficiency (PE) was calculated by dividing counted colonies by cells seeded (minushi *et* a*l*., 2005). Evaluation of survival fractions was performed using Hall 2006 formula as follow:

$$Surviving\ fraction\ (S) = \frac{Colonies\ counted}{Cells\ seeded \times PE/100}$$

2.7 Micronucleus assay:

Immediately after irradiation cells were harvested by trypsinisation by removing the medium and the flasks were washed by 1ml phosphate buffer saline, then cells were diluted and counted by cells coulter counter, and plated into new sterilized T 25-flasks at about 30,000 cells/flask, containing complete medium with cytochalasin-B (final concentration of (3µg/ml) and incubated at 37ºC for 24 hours. After 24 hours the medium was removed and cells were rinsed with PBS, fixed with 70% ethanol for 7minutes, dried overnight and stained with 10% GIEMSA solution for about 15 minutes, washed by tap water three times and dried overnight. The flasks were sliced to small slides and covered with kanada balsam oil and covering glass and scored using 100 x magnifications, three slides were scored for each dose, a minimum of 10^3 binucleated cells were scored using per slide a ZEISS light microscope under oil (100× magnification). Following the scoring criteria described in details by Fenech *et* a*l*., 2003 as follows:

Cells should be binucleated, and the two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary, they should be approximately equal in size, staining pattern and staining intensity, the two nuclei within a BN cell may be attached by a fine nucleoplasmic bridge which is no wider than one-fourth of the largest nuclear diameter, the two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable, also the cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

MN are scored according to the following criteria:

- Micronuclei are morphologically identical to but smaller than the main nuclei, moreover, they also have the following characteristics.
    - The diameter of micronuclei usually varied between 1/16 and 1/3 of the mean diameter of the main nuclei which corresponds to 1/256 and 1/9 of the area of one of the main nuclei in a binucleated cell respectively.
    - They are round or oval in shape.

- They are non-refractile and they can therefore be readily distinguishable from artifacts such as staining particles.
- They are not linked or connected to the main nuclei.
- They may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- They usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

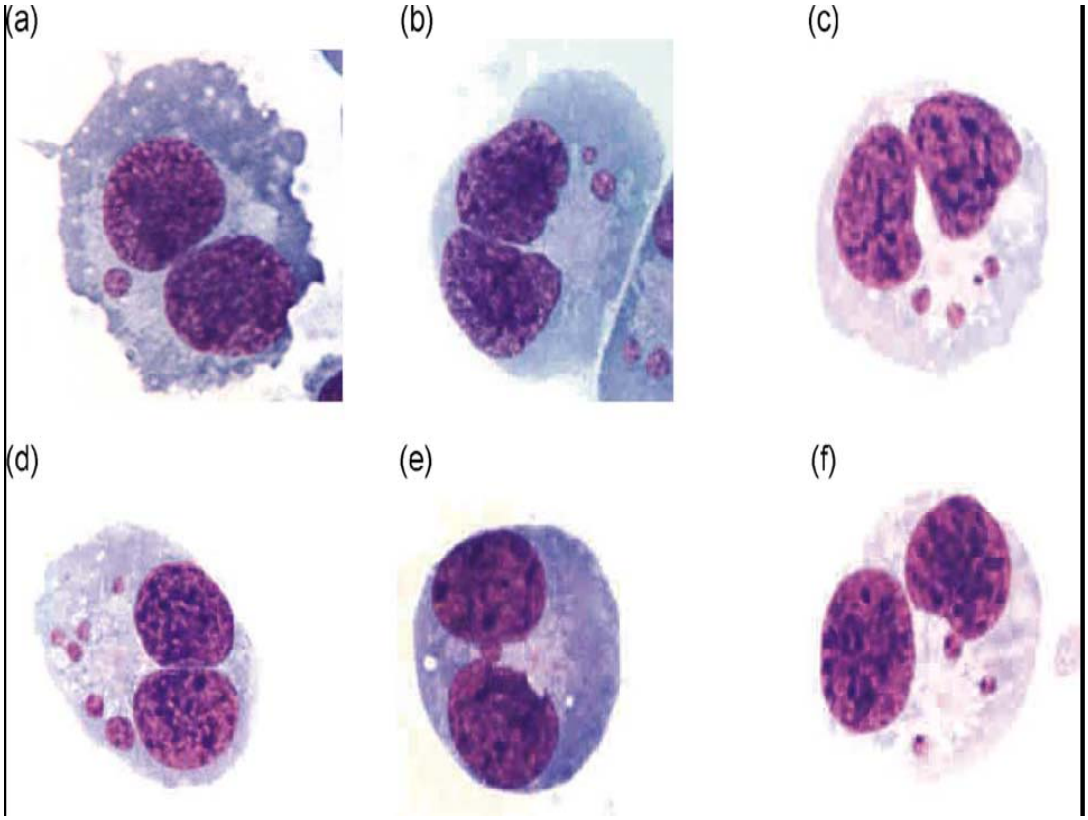

Figure (2.1): Photomicrographs of typical binucleated cells (BN) with micronuclei (MNi) from (Fenech *et* a*l*., 2003).

2.8 Statistical analysis:

Karyotype analysis results was presented as histogram using Microsoft excel programme. Surviving fraction results was presented as curve using Microsoft excel programme. Micronuclei MN frequency data were analyzed using (SPSS Version 16) Pearson correlation test to evaluate the effects of the two different energy photon-beams and the results were shown in curve fashion.

Chapter three

Results

In this study the radiobiological effect of two different energy photon beams (6 and 15MV) were evaluated using the biological system known as Chinese hamster V79 cell line to measure the survival curve and micronuclei induction in lung fibroblasts cell line that was cultured in Eagle's Minimal Essential Medium (MEM) supplemented with foetal calf serum, glutamine, gentamycine and HEPES and incubated at 37ºC in humidified 2% CO_2 atmosphere. Cells were exposed to 0.25, 0.5, 1.0, 2.0, 4.0 and 7.0 Gy using 6MV and 15MV photon beam energies from radiotherapy linear accelerator. This study aimed to determine the surviving fraction as a main radiobiological end point for two different energy photon beams, 6 and 15 MV. The late effects was investigated using MN assay at scattering radiations for different treatment modalities and to investigate the effect of photoneutron dose resulted from high energy photon beam (15MV) with special consideration to the late effects of radiotherapy.

3.1 Plating efficiency:

The V79 Chinese hamster cell line has been widely used as a test system in toxicology for many years. The plating efficiency of V79 cell line was performed following hall *et a*l 2006 procedure as mentioned above and the plating efficiency was calculated and found to be 95 % and was agree with data reported by (Simi *et al.,*1999).

3.2 Karyotype analysis:

Karyotype analysis is an approach that permits comprehensive examination of cellular DNA content based on quantitative analysis of short fragments of genomic DNA. Karyotype analysis may be used to study chromosomal aberration and genetic abnormality i.e. trisomy, deletion, ring chromosomes. To study the general features of Chinese hamster V79 cell line the karyotype analysis was performed following Sargent *et al* (1996) method as mentioned previously with minor modification and the ideal number of chromosomes for V79 cells was found to be (2n = 22 chromosomes) after counting 60 mitosis as shown in figure 3.1. The majority of cells, about 84%

equivalents to 50.4 cells out of 60 counted mitosis carrying 22 chromosomes as shown in figure 3.1.

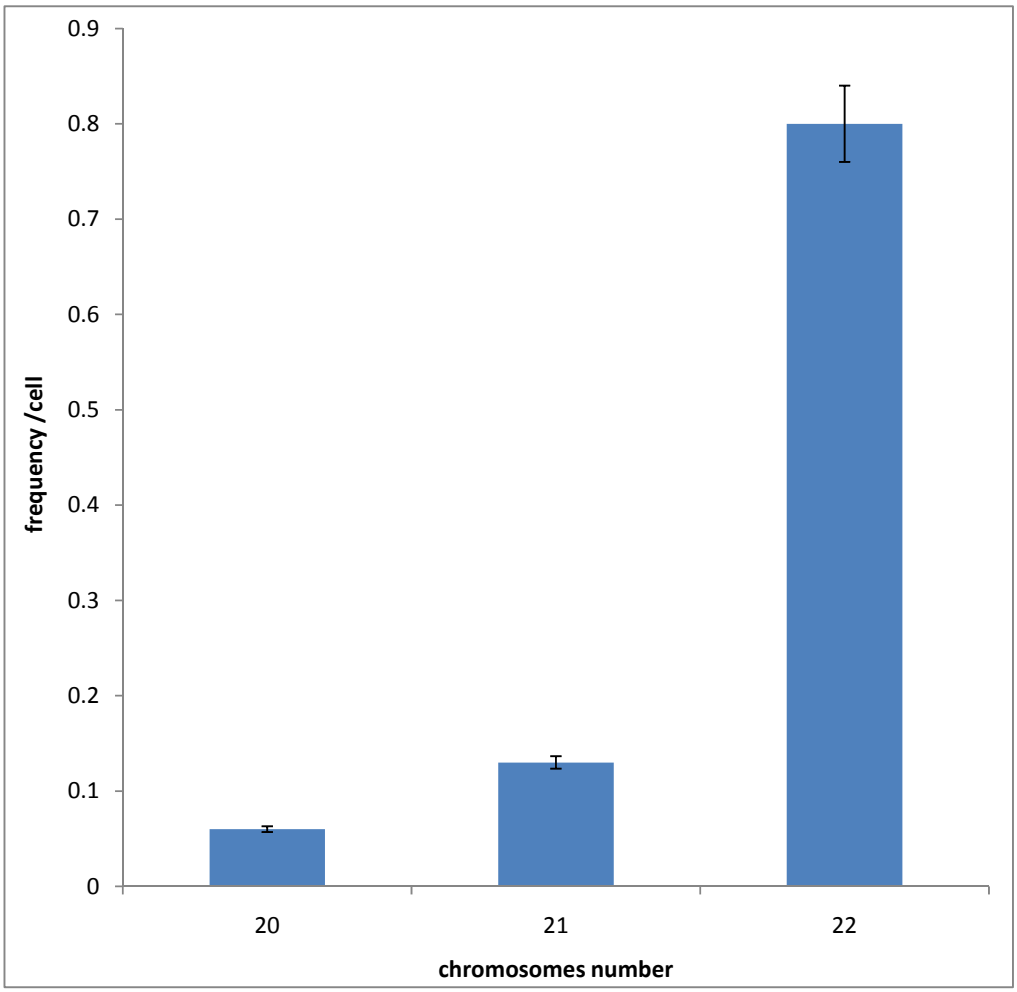

Figure (3.1): The ideal number of chromosomes in V79 cells was (2n=22chromosome).

3.3 Cell survival curve:

The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone. This cell is then said to be clonogenic. A cell survival curve is therefore defined as a relationship between the dose of the agent used to produce an insult and the fraction of cells retaining their ability to reproduce. Although clonogenic cell survival assays were initially described for studying the effects of radiation on cells and have played an essential role in radiobiology, they are now widely used to examine the effects of agents with potential applications in the clinic. In this study survival curve has been carried out in Chinese hamster V79 cell line growing in culture. One can use cell lines from various origins including humans and rodents, these cells can be neoplastic or normal. Survival curves have wide application in evaluating the reproductive integrity of different cells. To assess the surviving fraction colony survival assay was performed according to minushi *et al* 2005 method as mentioned above and surviving fraction was plotted against the dose for both 6 and 15 MV. The results were shown in figure 3.2 the surviving fraction was negatively correlated with dose. For both of 6 and 15 MV photon beams energies as shown in figure 3.2.

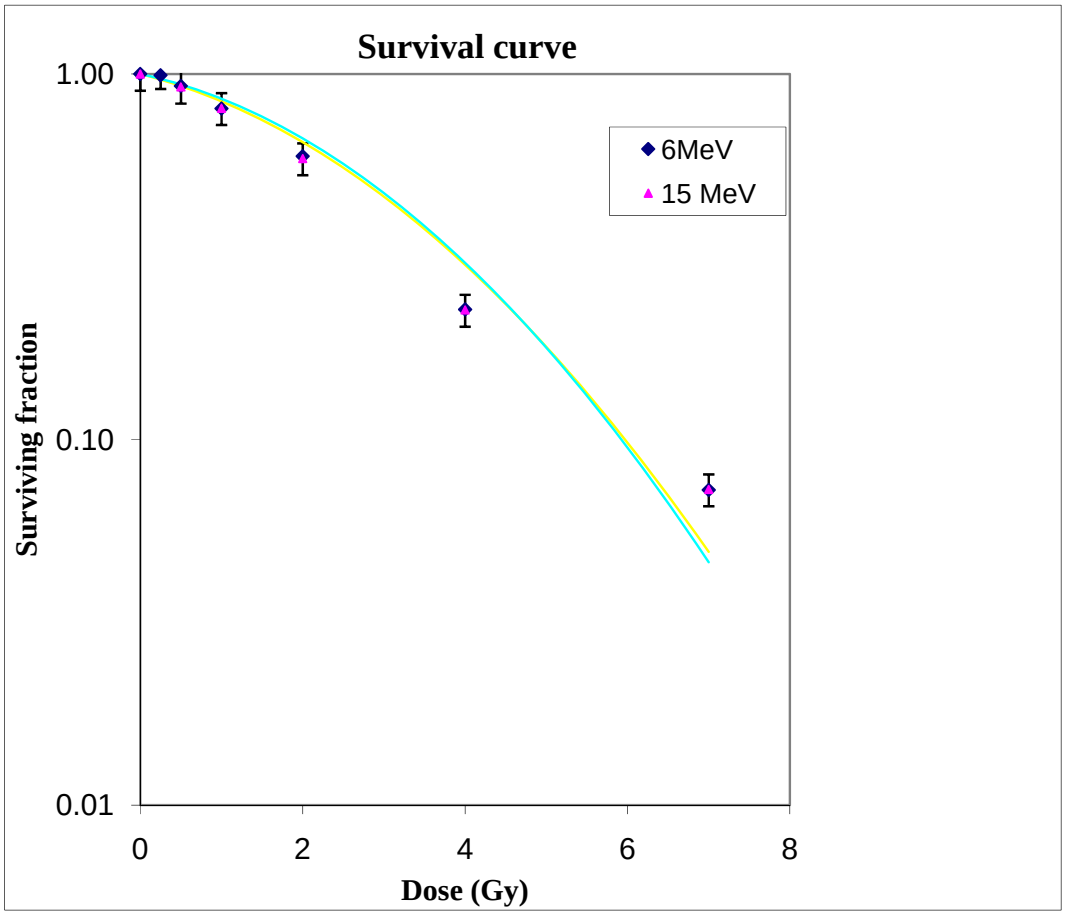

Figure (3.2): Surviving fraction versus photon dose for V79 cells irradiated with the two different energy photon beams 6 and 15 MV with different dose rate.

3.3 Micronuclei (MN):

The data from a micronucleus assay are usually represented as the number of micronuclei per number of binucleated cells, making the results vary depending on the stage of the cell cycle. It was found that while the total number of binucleated cells and the total number of micronuclei increase with time of incubation in cytochalasin B, the ratio of binucleated cells with micronuclei per total number of scored binucleated cells remains more or less constant. In this study micronucleus assay was carried out and the frequency of micronuclei MN was evaluated after scoring of 3000 cells/dose, the cell cycle kinetic data indicated that the frequency of micronuclei increased with increasing photon dose, and significantly elevated with increased photon energy as shown in figure (3.3).

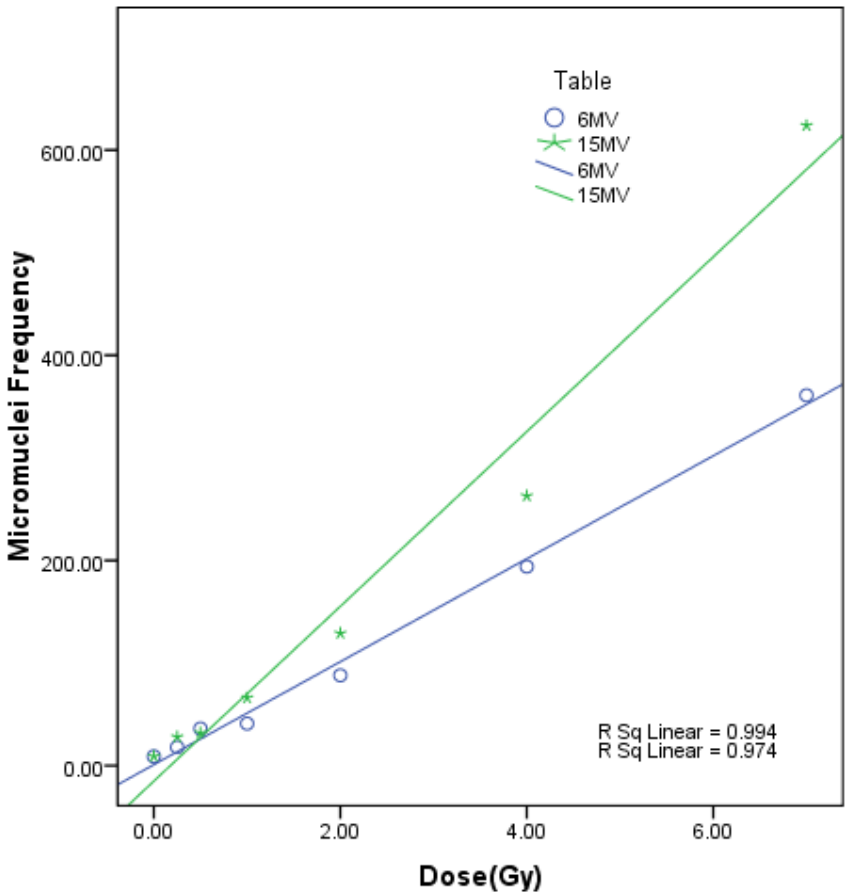

Figure (3.3): The frequency of micronuclei in Chinese hamster V79 cell line after irradiation with two different energy photon beams 6and 15MV.

Chapter Four

Discussion

The most common method of representing results from the micronucleus assay is to count the number of micronuclei per 1000 binucleated cells. Since ionizing radiation causes a dose-dependent mitotic delay in exposed cells as the dose increases. It becomes necessary to incubate cells with cytochalasin-B for prolonged periods to obtain a sample with a sufficient number of binucleated cells, following exposure to various radiation doses. Dose-response parameters were determined by measurement of surviving fraction and MN assay CYB method. The biological system used in this study was Chinese Hamster lung fibroblast V79 cell line that was cultured in Eagle's minimal essential medium (MEM), supplemented with foetal calf serum, glutamine, gentamycine and HEPES, cells were irradiated and reseeded with suitable numbers for both survival curve analysis and micronuclei measurement in new culture medium, incubated, stained and counted for survival curve and micronuclei. Cell survival was evaluated and micronuclei were measured for each dose and specified photon beam energies. Relation between dose and effect was evaluated using Microsoft excel programme for survival curve and bearson correlation test for micronuclei.

4.1 Plating efficiency (PE):

The plating efficiency of a cell line may give information about the cell line behavior, their growth in medium, their doubling time and cell cycle duration. PE is very important in calculation of surviving fraction (Anderson *et al.,* 2000). To study the viability of the V79 cells, the plating efficiency was carried out using Hall *et al* 2006 method, the plating efficiency was found to be 95% which agrees with the previously reported data by Pathak *et al* in 2007, that he found the plating efficiency was varied 85-95, variation in plating efficiency may be due to cells trypsinisation, poor culture adaptation and scoring experience.

4.2 karyotype analysis:

Karyotype analysis is essential for comprehensive examination of cellular DNA content based on quantitative analysis of short fragments of genomic DNA (Kawata *et al*., 2004). Karyotype analysis may be used to study chromosomal aberration and genetic abnormality i.e. trisomy deletion, ring chromosomes (Lee *et al.,* 2005). As shown in Figure 3.1**,** and after applying Sargent *et al* 1996 with minor modification the ideal number of chromosomes in V79 cells was found to be (2n = 22 chromosomes) after counting of 60 cells, as shown in the column (3.1) there are some differences in the chromosomes numbers the variation in chromosomal number in some cell in this study may be due to overlapping of mitotic cells. This result is in agreement with previously reported data for V79 cell lines by Trott and Teibe in 1998.

4.3 Cell survival curve:

The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone, this cell is then said to be clonogenic. A cell survival curve is therefore defined as a relationship between the dose of the agent used to produce an insult and the fraction of cells retaining their ability to reproduce. Although clonogenic cell survival assays were initially described for studying the effects of radiation on cells and have played an essential role in radiobiology, they are now widely used to examine the effects of agents with potential applications in the clinic (Munshi *et al*., 2005). In this study survival curve has been carried out in Chinese hamster V79 cell line growing in culture. One can use cell lines from various origins including humans and rodents, these cells can be neoplastic or normal. Survival curve has a wide application in the evaluation of reproductive integrity of different cells. Figure 3.3 shows the dose-effect survival curve which depicts similar effect of both low and high photon energies. The results of this study show negative correlation between cell surviving fraction with radiation dose and this finding is in an agreement with data reported by Trott and Teibe in 1998, that ionizing radiations induce apoptosis and genomic instability which leads to decrease colony forming ability in V79 cell line. Our study reasserts the fact that ionizing radiation is the most effective cause of cell death that mainly occurs by activation of

apoptosis through the activation of many cellular factors. The results of the present study agree with previously reported data by Almasan (2000).

4.4 Micronuclei (MN):

Radiation-induced MN arises mainly from a centric chromosomal fragment, although a significant proportion appears to originate from the entire chromosomes (Schuler et al., 1997). Studies on the mechanism of radiation-induced MN have confirmed that MN originates from a centric fragment. Acentric fragments result predominantly due to terminal deletions. Dicentrics are mostly accompanied by acentric fragments. The minutes and double minutes are thought to arise due to the amplification of a certain region in the genome. Radiation also produces a dramatic cytotoxic effect as evidenced by slow cell division and or lack of metaphase cells. One possible reason for such an effect is that cells may be taking time to repair the damaged chromosomes. Figure (3.3) shows the dose response curve for micronuclei in V79 cells irradiated with two different photon energies 6 MV (blue mark) and 15 MV (green mark). The curve shows linear correlation of micronuclei frequency with dose. The results in this study agree with Medvedeva (2004) who concluded that the fraction of binucleated cells containing micronuclei depends on the amount of radiation dose.

The result of this study may confirm the data reported by trot and Teibe in 1998 that in Chinese hamster V79 cells ionizing radiation induced genomic instability resulted in a persistently increased frequency of micronuclei.

This study shows significant high frequency of micronuclei, for 15 MV, this may be due to increase in photon energy and there is large variation in micronuclei frequency after exposure to high energy photon beams. This may highly suggest the presence of small dose of photoneutrons, as it is well known photoneutrons have high relative biological effectiveness (RBE) even with low dose which may lead to significant biological effects (Hall *et al*., 1995). The photoneutrons produced by the interaction of high energy photon with high Z numbers metals in the linear accelerator head, the production of photoneutrons starts with threshold of about 8 MV, As the beam energies increase (>8 MeV), the undesirable photoneutron doses also increases and thus enhances the risk of second malignancies, the contributed dose is noticeable when concerning about neighboring critical organs, examples the dose of gonads in radiotherapy of intestinal

cancer. Presently, studies of photoneutron in literatures mostly lay stress on spatial distribution of neutrons in therapeutic chambers. However, actual accepted neutron dose of the workers around the therapeutic chamber as well as the internal distribution of the patients is seldom probed. In order to optimize treatment conditions and avoid unnecessary radiation injury in patient care, the dose from photoneutron investigation is imperative to provide support for the field of health physics and medical physics to accurately estimate received dose of the patients (Lin *et al*., 2007). These photoneutrons have very high linear energy transfer and high biological effect in proportion to photon (Errico *et al*., 2001). This leads to high rate of DNA damage and then high frequency of micronuclei as appears after applying 15 MV, these data support the results reported by Zanini *et al* 2004. He reported that during radiotherapy, when the photon energy exceeds 10 MV the patient receives an undesired dose due to photoneutron production in the accelerator head. With high energy photon beams that may include photon with more than 15 MV, in these cases patients may be exposed to more dose of photoneutrons. The neutron dose equivalent within the patient plays an important role in determining the risk of second malignancies particularly in long survivors for example childhood patients who may live more than twenty years after radiotherapy treatment. This study supports the use of micronuclei as biodosimetric tool for the evaluation of biological effects of ionizing, our background micronuclei data for V79 cell lines agree with the results published previously by Ellard *et* a*l* 1991, who described spontaneous micronucleated cell frequency for different V79 cell lines as varying between 9 and 26.1 per 1000 cells, and the corresponding percentages of binucleated cells ranging from 60.7 to 83.3%.

Conclusions

The cells exposed to15MV photon beam energy were found to be with significantly increased micronuclei frequency compared with cells exposed to 6MV photon beam energy.

The results of this study may support the hypothesis that the Cytokinesis-block MN assay is a useful tool for the assessment of carcinogenic late effect of ionizing radiation exposure as well as the evaluation of other clastogenic compounds such as smoking and chemical exposure.

Recommendations

More evaluation of radiation protection measures may be needed in radiotherapy patients especially when applying photons with high energy and more care should be devoted to the childhood cancer patients.

Fortunately we are establishing a radiobiology laboratory for the first time in Sudan, to support the radiation protection through the application of biodosimetry which will greatly contribute to the improvement of radiotherapy conditions and may decrease the late effects of scattering radiation during radiotherapy.

Further survival curve studies may be performed for evaluation of treatment dose and assessment of chemo-therapeutic agents.

The micronucleus assay may be applied on patient's blood samples after radiotherapy to assess the potentiality of late effects (secondary cancer) of radiotherapy.

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