MULTI TARGET ANALYSIS OF HELA CELLS RADIOSENSITIVITY

by

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CERTIFICATE

This is to certify the dissertation entitled

Multi Target Analysis of HeLa Cells Radiosensitivity

Is the bona fide record of research work done by

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LIST OF ABBREVATIONS

Gy – Gray

MT – Multi Target

LQ – Linear Quadratic

M – Mitosis

S – Synthesis

LET – Linear Energy Transfer

HPV18 - Human Papilloma Virus 18

MTSH - Multi Target- Single Hit

CO_2- Carbon Dioxide

LINAC- Linear Accelerator

DPBS - Dulbecco's Phosphate Buffered Saline

DMEM - Dulbecco's Modified Eangle Medium

PBS - Phosphate-Buffer Saline

SLD - Sublethal Damage

ABSTRACT

Introduction: The main objective of this study is to investigate the radiosensitivity of HeLa cells based on analysis using multi target model.

Methods: The cell irradiations were performed using photon beam of energy 6 MV, 10 MV and electron beam of energy 6 MeV, 15 MeV. Samples were setup in full scatter condition using solid water phantom and bolus. Each sample was exposed to doses range from 1 to 10 Gy. Clonogenic assay were performed to obtain the cell survival curve data and the data were later analysis using Multi Target (MT) model. Analysis using Linear Quadratic (LQ) was also done for comparison.

Results and Discussion: From the result, it shows that the survival curve for electron was steeper compared to photon beam. Therefore, HeLa cells were more radiosensitive to electron beam compared to photon beam. The MT model shows dominant reason of death cell due to multiple events cell killing which represents by D_0. The value for D_0 for 6 MV, 10 MV, 6 MeV and 15 MeV were 2.42385, 3.39997, 2.42385, and 4022.75834 respectively. The MT model was found to be fitted better compare to LQ model for different energies and especially for high dose.

Conclusion: HeLa cell is more radiosensitive toward photon beam than electron beam for MT Model while for the LQ model, cells were more radiosensitive to electron beam compare to photon beam. The Multi Target model describe the cell survive curve for high dose better than Linear Quadratic model.

ABSTRAK

Pengenalan: Tujuan utama kajian ini adalah untuk mengkaji radiosensitiviti sel HeLa berdasarkan model berbagai sasaran.

Kaedah: Sel kanser telah didedahkan kepada radiasi menggunakan 6 MV and 10 MV alur foton dan 6 MeV dan 15 MeV alur elektron. Sampel disediakan dengan menggunakan bolus dan 'phantom' air pepejal. Setiap sampel didedahkan kepada dos dari 1 Gy sehingga 10 Gy. Teknik clonogenic dilakukan untuk mendapatkan keluk sel survival dan data tersebut kemudian dianalisa berdasarkan model berbagai sasaran.

Keputusan dan perbincangan: Daripada keputusan kajian, lekuk sel survival alur elektron lebih curam berbanding alur foton. Oleh itu, sel HeLa lebih radiosensitif kepada alur elektron berbanding alur foton. Model berbagai sasaran menunjukkan sebab dominan sel mati kerana peristiwa berganda pembunuhan sel yang diwakili oleh D_0. Nilai D_0 untuk 6 MV, 10 MV, 6 MeV, dan 15 MeV adalah 2.42385, 3.39997, 2.42385, dan 4022.75834. Model berbagai sasaran didapati lebih sesuai berbanding model linear kuadratik untuk tenaga yang berbeza dan terutama dos yang tinggi.

Kesimpulan: Sel HeLa lebih sensitif kepada alur foton berbanding alur electron untuk model berbagai sasaran manakala bagi model linear kuadratik, sel lebih radiosensitif kepada alur elektron berbanding alur foton. Model multi sasaran menjelaskan lekuk sel survival untuk dos tinggi lebih baik dari model linear kuadratik.

CHAPTER 1

INTRODUCTION

1.1 Radiotherapy

Radiotherapy is commonly used for cancer treatment using high energy radiation which is usually combined with chemotherapy and surgery. There are two types of radiotherapy that are external and internal radiotherapy. External radiotherapy is radiation source comes from a machine which is outside the body while internal radiotherapy is radiation source comes from implants radioactive material in form of seeds, wires, or tubes inside the body for certain period. For this study, the external radiotherapy is used for cancer treatment where the treatment is delivered using a machine called a linear accelerator. The linear accelerator is used to treat the cancer for all parts or organ of the body by delivering high energy of x-rays or photons to the treatment areas. The linear accelerator will produce ionizing radiation to kill the cancer cells. The ionizing radiation will damage the DNA of the cells directly or creates charged particles called free radicals that damage the DNA of the cells which cause the cancer cells stop growing and die. However, the normal tissue surrounding treatment areas will be damaged but they can usually repair themselves. The normal tissue also can be spared by using blocks or multi leaf collimator when shaping the beam.

1.2 Radiobiology

Radiobiology is defined in general terms as the science that evaluates the effects of radiation in living organisms. In the field of radiation oncology, it is defined as the science that investigates the interactions between ionizing radiation and living systems, and the consequences of these interactions (Beyzadeoglu, et al., 2010).

Figure 1.1 shows the cell cycle phase which is important for understanding the relationship between cells and radiation therapy. Radiation usually will firstly kill the cells at phase that are actively dividing or called cell proliferation. The cell proliferation cycle is at phase mitosis (M), where division takes place and the period of DNA synthesis (S). Besides that, the term radiosensitivity is used to describes how likely the cell is to be damaged by radiation. In general, cells are most radiosensitive in the M and G_2 phases, and most resistant in the late S phase (Suntharalingam, et al., 2005).

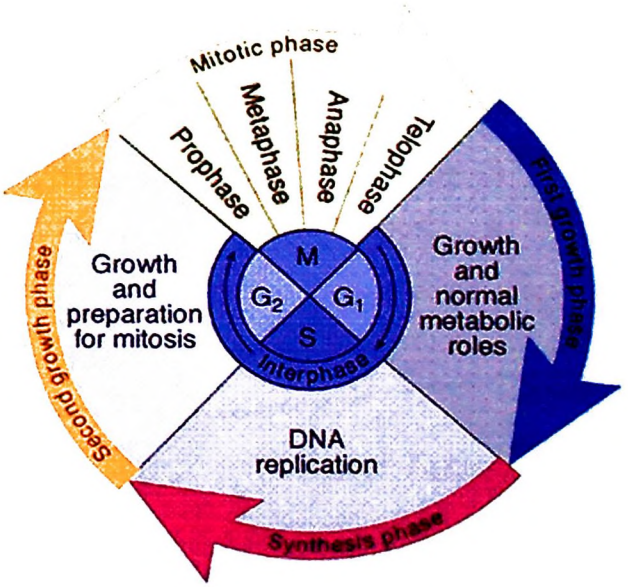

Figure 1.1: Cell cycle phases
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Radiation is used to kill cancer cells and shrink tumors while genes control how cells grow and divide. Therefore, the radiation works by damaging the DNA in cells. When radiation damages the genes of cancer cells, they can't grow and divide any more. Then, the cells will die. There are two ways how radiations damage the DNA that are by direct and indirect action.

Figure 1.2 shows the direct and indirect action of radiation towards DNA. In direct action, the radiation interacts directly with the critical target in the cell. The biological damage occurs when the atoms of the target itself may be ionized or excited through Coulomb interactions (Suntharalingam, et al., 2005). Direct action usually occurs due to high linear energy transfer (LET) radiation such as neutrons and α particles. In indirect action, the radiation interacts with other molecules and atoms within the cell to produce free radicals. By diffusion, free radical will cause the biological damage (Suntharalingam, et al., 2005). The indirect action usually happened due to low LET radiation.

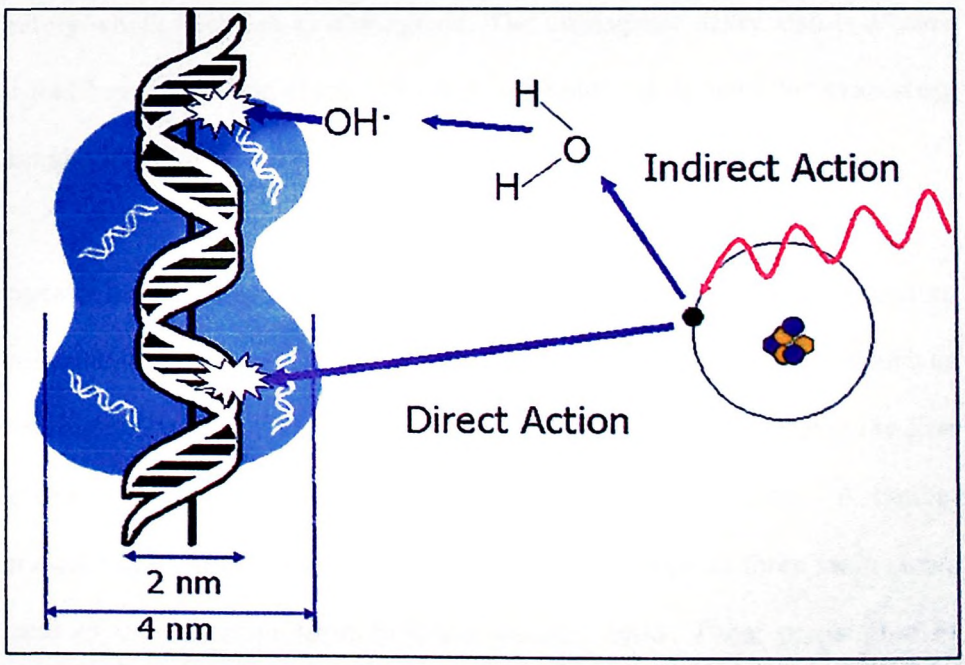

Figure 1.2: Direct and indirect action of radiation towards DNA
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1.3 HeLa cells

HeLa cell is the human cervical cancer cells where it becomes cancerous due to infection with human papilloma virus 18 (HPV18). The cultured of the cells start in 1951 and the first human cancer cell to be continuously grown in culture for research. HeLa cell is appear to be immortal because the cell multiply and grow quickly in an uncontrolled way, compared to normal cells with right conditions, nutrients and space. It also can spread and infect other cells (The Cell, 2011). Nowadays, HeLa cells are widely used in medical research.

1.4 Clonogenic Assays

Clonogenic assay is defined as an in vitro cell survival assay based on the ability of a single cell to grow into a colony (Franken, et al., 2006). The cell that grows into colony is a surviving cell that maintains its reproductive integrity and proliferates almost indefinitely which is called as clonogenic. The clonogenic assay also is a basic technique that had been described since 1956 and has been widely used for evaluating the radiation sensitivity of different cell lines (Rafehi et al., 2011).

The clonogenic assay enables an assessment of the differences in reproductive between control untreated cells and cells that have undergone various treatments such as exposure to ionising radiation. In addition, the initial landmark study generated the first radiation-dose response curve for X-ray irradiated HeLa cells in culture. A typical clonogenic survival experiment using mammalian cells lines involves three main steps. Firstly, treatment of the cell monolayer in tissue culture flasks. Then, preparation of single cell suspensions and plating an appropriate number of cells in petri dishes and

lastly, fixing and staining colonies following a relevant incubation period, which could range from one to three weeks, depending on the type of cell (Rafehi et al., 2011).

1.5 Cell Survival Curve

A cell survival curve describes the relationship between the surviving fraction of cells and the absorbed dose. The surviving fraction of cells is obtained from the clonogenic assay technique based on the colony form. The survival curves of mammalian cells also can be used to obtain direct information on their response to radiation. In addition, the different type of radiation influences the shape of the cell survival curves.

1.6 Multi Target- Single Hit (MTSH) Model

Target theory is one of the essential concepts for understanding radiation biology. Although many complex interpretations of target theory have been developed, its fundamental principle is that inactivation of the target inside an organism by radiation results in the organism's death (Nomiya, 2013).

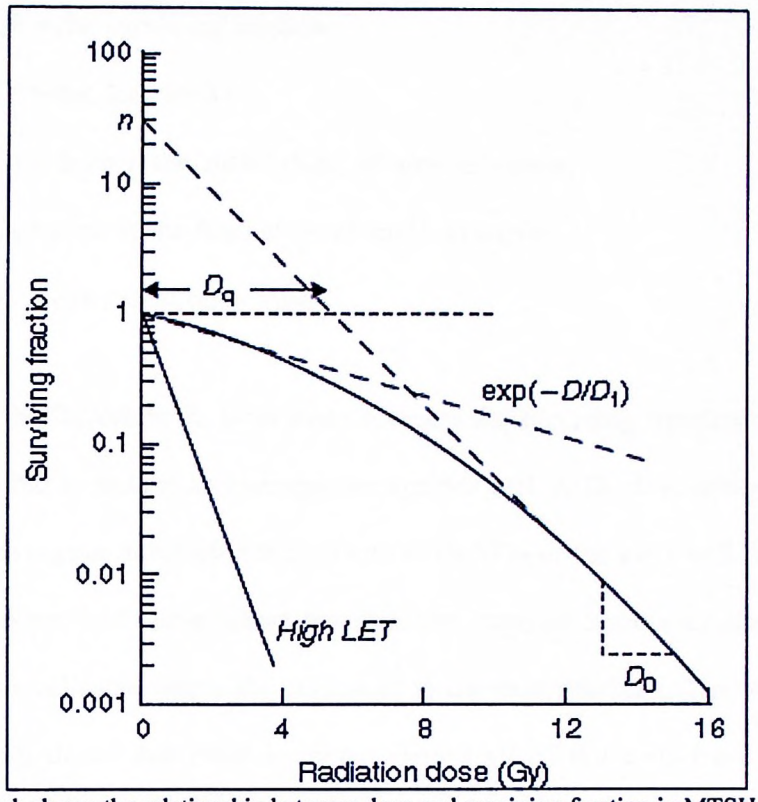

Figure 1.3: The graph shows the relationship between dose and surviving fraction in MTSH model. (Joiner, M., & Kogel, A.V.D. (2009). Basic clinical radiobiology (4th ed.). Edward Arnold: United Kingdom).

Figure 1.3 is the graph shows the relationship between dose and surviving fraction in MTSH model. Multi target (MT) model defines that there are more targets than just one in an organism, and inactivation of all the targets leads to death of the organism (Nomiya, 2013). MTSH is defined as the required multiple targets per cell and only one of the targets need to be hit to kill the cell (Beyzadeoglu, et al., 2012). Even though MT model is mentioned in many textbooks of radiation biology, it is rarely used in present clinical and basic studies. The surviving fraction of MTSH model is expressed as

$$SF = e^{-D/D_1}\left[1 - \left(1 - e^{-D/D_0}\right)^n\right]$$

Where, SF = the surviving fraction

D = the dose in Gy

D_1 = dose at the initial slope of survival curve

D_0 = dose at the final slope of survival curve

n = extrapolation number

In addition, the D_0 also is the dose that decreases the surviving fraction to 37%. This is the dose required to induce an average damage per cell. A D_0 dose always kills 63% of the cells in the region in which it is applied, while 37% of the cells will survive. D_q is the region of the survival curve where the shoulder starts or known as quasi-threshold dose. This region indicates where the cells start to die exponentially. The value n show the number of D_0 doses that must be given before all of the cells have been killed (Beyzadeoglu, et al., 2012). MT models also seem to be more reliable at 6 Gy or higher doses (Iwata et al., 2013).

1.7 Linear Quadratic Model

Linear Quadratic (LQ) model is developed by Douglas and Fowler in 1972. The LQ model is used widely for both experimental and clinical radiobiology and generally works well in describing responses to radiation in vitro and in vivo. LQ model also are used for to formulate equivalent fractionation schemes, to calculate additional doses after breaks from radiotherapy, and to get information on acute and late responses (Beyzadeoglu, et al., 2012).

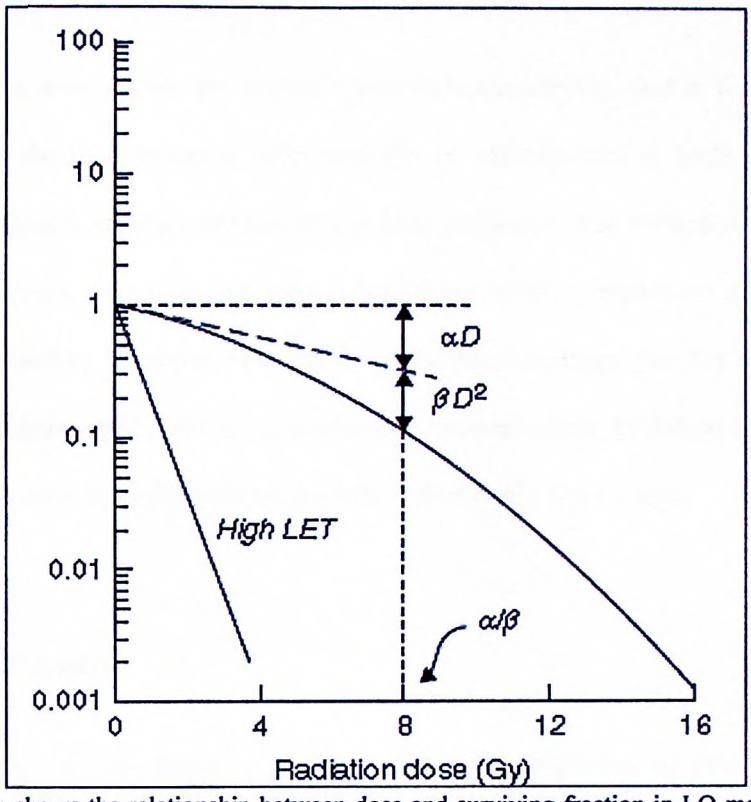

Figure 1.4: The graph shows the relationship between dose and surviving fraction in LQ model. (Joiner, M., & Kogel, A.V.D. (2009). Basic clinical radiobiology (4th ed.). Edward Arnold: United Kingdom).

Based on Figure 1.4, it shows that in LQ model, it was assumed that cell death is due to ionizing radiation that has two components. The first component is linear component which is directly proportional to dose while the second component is quadratic component which is directly proportional to the square of the dose. The surviving fraction of LQ model is expressed as

$$SF = E^{-(\alpha D + \beta D^2)}$$

Where, SF = the surviving fraction

D = the dose in Gy

α = is a constant describing the linear component of cell killing

β = is a smaller constant describing the quadratic component of cell killing

The value α also shows the intrinsic cell radiosensitivity, and it is the natural logarithm (log) of the proportion of cells that die or will die due to their inability to repair radiation-induced damage per Gy of ionizing radiation. For valueβ, it shows the cell repair mechanisms, and it is the natural logarithm of the proportion of repairable cells due to their ability to repair the radiation-induced damage per Gy of ionizing radiation (Beyzadeoglu, et al., 2012). According to research done by Iwata et al (2013), the LQ model may only be applicable to fractional doses of 5 Gy or less.

1.8 Significance of Study

The significance of this study is to know the radiosensitivity of HeLa cells for different energy since different type of cells will shows different response to the radiation. The radiosensitivity and radiobiology response of HeLa cells are evaluated based on MT model. The cell survival curve plotted based on MT model is then compared to LQ model. From this comparison we can see which model can describe better about the radiosensitivity and radiobiological response of the cells for different energy in range of 1 to 10 Gy of dose.

CHAPTER 2

LITERATURE REVIEW

HeLa cells are the cervical cancer cell of a young African-American woman, Henrietta Lacks. Henrietta Lacks died in 1951 of an aggressive adenocarcinoma of the cervix. The cells were first cultured in February 1951 by Drs. George and Margaret Gey and become the first human cell line grew rapidly in cell culture. The characteristic of HeLa cells were a robust, immortal cell line, easily propagated over generations in culture and can contaminate other cell line. Interspecies cross-contamination with HeLa, easier to detect than intraspecies contamination, was described in the early 1960s (Lucey et al., 2009). Despite the passing of nearly 50 years since the problem first surfaced of HeLa cell contamination of tissue cultures, HeLa still widely used by researchers around the world.

The cell survival curve was used in the research done by Puck and Marcus (1956) to describe the effects of high energy irradiation on the reproductive capacity of single HeLa cells by plotting the graph of number of cells colony versus dose. According to the graph plotted by Puck and Marcus (1956), it defines the relationship between the radiation dose and the proportion of cells that survive. In addition, the cell culture assay technique was recommended by Markis (2010) in order to obtain the cell survival curve and the surviving fraction can be calculated by counting the cell colony form after staining.

There are several models used to analysis the cell survival curve. In this study only Multi Target- Single Hit (MTSH) is used and then compared with Linear Quadratic (LQ) models. Alpen (1997) stated six properties cell survival curve based on MTSH function. The first properties is each log curve becomes a straight line for surviving fraction below about 0.1, at which time the shoulder region has no more effect on the curvature. The second properties is the extrapolation of this straight line portion of the plot back to the zero dose ordinate yields a value for n, the target multiplicity. However, except for the case of n = 1, in which circumstances the relationship degenerates to single-hit form, every curve has a shoulder that increase in breadth with the multiplicity, n. The curves for any greater than 1 will have zero slopes at zero doses. The fifth properties is q has the dimensions and nature of the inactivation coefficient as in single-hit case and last properties is 1/q is the dose for $1/e$ (37%) survival in the linear portion of the survival plot, and it is called the D_0. The D_0 has the dimensions of dose.

Even though most textbooks mention about the MT model, this model is not widely used due to some limitation. The first limitation is regarding the value of D_0 where the determination of the value D_0 shows that it is not constant at it is measured at lower and lower survival levels especially for mammalian cell lines. Generally the D_0 tends to decrease as the survival level decreases (Alpen, 1997). According to Alpen (1997) review, the most convincing evidence for the changing D_0 is by Hall et al. (1972), who found a significant and continuous decrease in D_0 as survival was followed through many decades of survival for a mammalian cell line. The second limitation is the initial slope of survival curve for the MTSH model predicts that a zero slope will be found at zero doses. This is a very difficult value to establish experimentally, but the evidence continuous to accumulate that, for most systems, the initial slope is significantly less than zeros (Alpen, 1997).

Despite the limitation of MT model, it is useful as an alternative description of clonogenic survival as a function of radiation dose. This classical model also represents a straight line at high doses, which is not supported by the mechanism of the underlying radiobiological processes but it is still valuable because MT model fits the empirical data well, especially in the high-dose range. Iwata et al. (2012) states that conversion with the MT models is not easy in clinical practice since there are many parameters which generally cannot be determined. However, in his study the used of MT models for biological dose estimation was possible because the Mathematica software was used.

LQ model was proposed by Chadwick and Leenhouts in 1981 in order to correct the deficiencies of the MT model. According to Alpen (1997) there are five assumptions regarding LQ model. Firstly he assumed that LQ model have certain critical molecules in the cell which is essential for the survival of the reproductive function of the cell. This is a refinement of the definition of the target or targets. Secondly, these critical molecules are assumed to be double-stranded DNA, and the critical damage is assumed to be a double-strand break in the DNA molecule. Other than that, the action of radiation, either direct or indirect, is considered to be rupture of molecular bond in DNA strands (lesion). The fourth assumption is the lesions in DNA are capable express the varying degrees of repair under certain conditions of being repaired, and modifications of radiobiological effects. Lastly, the repair process of DNA includes physiochemical recombination, charge transfer process, chemical restitution and enzymatic repair (Alpen, 1997). Fertil and Malaise (1985) found that that human cell survival curves could be described by the LQ model. The low α-value which is indicating resistance in the low dose range is associated with high P-value. The exponential survival curve

shows the high radiosensitivity of cells while shouldered survival curve represents the poor radiosensitivity (Fertil and Malaise, 1985).

There are several studies that compare different models to analyze the cell survival curve. One of the studies comparing the different model was done by Iwata et al. (2012). From the study, it shows that MT models seem to be more reliable than the LQ model at 6 Gy or higher single doses. This result happen may because of the characteristics of the two models where in the high-dose range can be approximated by linear regression. The LQ model may only be applicable to fractional doses of 5 Gy or less. In this study, the applicability of the MT models to do dose conversion between single and hypofractionated doses, then, comparing them to the LQ model also was done. The results shows despite the MT model is a basic and classical linear model, it still fits the empirical data well at high doses compared to the LQ model (Iwata et al., 2012). Besides that, Fertil and Masaile (1985) found that parameters used by MT model that are n and D_0 do not accurately describe the differences in radiosensitivity that are a feature of the initial part of the survival curve. In LQ model, the use of the parameter α not only allows the differentiation between different cell lines, but also leads to consistency between different studies of a single cell line, as in the case of HeLa cells (Fertil & Masaile, 1985).

CHAPTER 3

OBJECTIVE OF THE STUDY

3.1 Aim

The aim of this study is to investigate the radiosensitivity of Hela cells based on multi target model.

3.2 Objectives

1. To measure the radiosensitivity of Hela cells for photon and electron beam
2. To evaluate the radiobiological response of Hela cells for photon beam and electron beam
3. To analyze the cell survival curve by using Multi Target (MT) Model
4. To compare the cell survival curve between Multi Target (MT) Model and Linear Quadratic (LQ) Model.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Cell Culture

4.1.1.1 HeLa cell

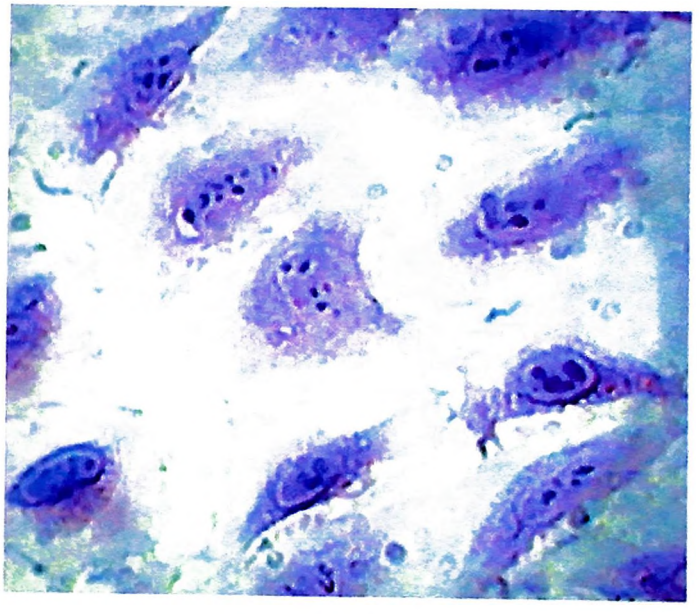

Figure 4.1: HeLa cell

HeLa cell is the first human cells continuously grown in cell culture. It is cervix cancer cell which have an epithelial-like morphology. This cell is widely used for cancer research because it can adapt to almost any environment, durable, and prolific compare to the other cell.

4.1.1.2 Culture flask

Figure 4.2: Cell culture flask

Culture flask provides a place for cell to growth and spread. There are several types of culture flask that are 25, 75 and 175 cm.

4.1.1.3 Pipette

Figure 4.3: Pipette

Pipette was used to withdraw and dispense cells, medium, and reagents. The appropriate size of pipettes was chosen based on the volume transferred. Then the pipette was set to the desired volume in order to get accurate measurements. Pipettes fit tips that are specific for the specific volumes. The tips was kept in color-coded sterile boxes and disposed after used.

4.1.1.4 Eppendorf tube

Figure 4.4: Eppendorf tube

Eppendorf tube was used to place the cells from culture flask that had been culture by using pipette. Eppendorf tube also have varies size and the size used was depend on the volume of cells.

4.1.1.5 Cell culture hood

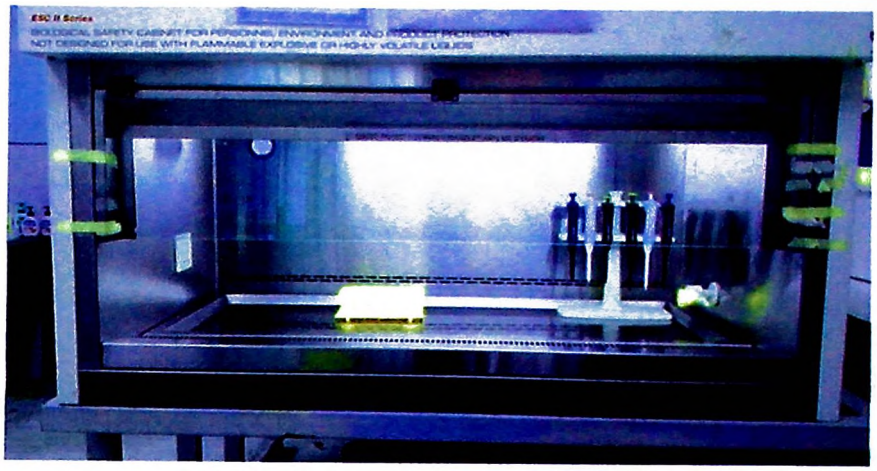

Figure 4.5: Cell culture hood

Cell culture hood was used to prepare the cell in clean working environment to prevent contamination of cell cultures. The hood consists of UV light which is important for sterilization of all materials that will be used. The UV lamp must be turned off before working in the hood to prevent exposure to hazardous UV light. 70% of ethanol must be used to wipe the surface of the hood and materials that will be used in the hood. After finished using the hood, ethanol was used once again to clean inside the hood.

4.1.1.6 Incubator (CO_2 Series Sheldon Mfg. Inc)

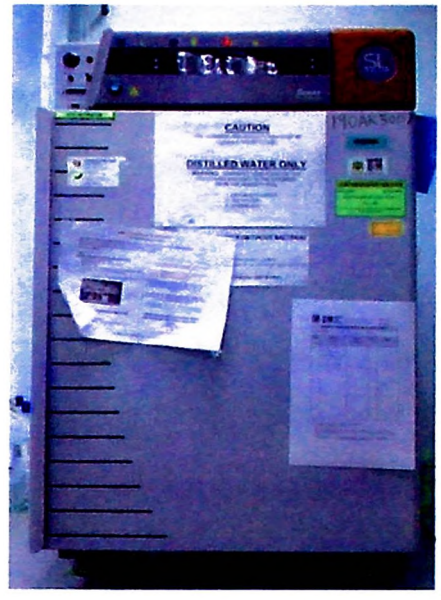

Figure 4.6: Incubator (CO_2 Series Sheldon Mfg. Inc)

Incubator is laboratory equipment that provides an environment suitable for the growth and maintains the cell cultures. The incubator main functions are maintains optimal temperature approximately about 37.0 ^{0}C, humidity of 35%, carbon dioxide (CO_2) level, and oxygen contain inside incubators.

4.1.1.7 Surgical gloves

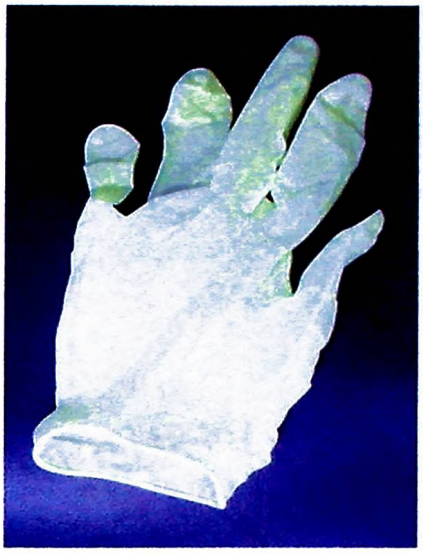

Figure 4.7: Surgical glove

Surgical gloves were used as protection for users from biohazards or other substances. The gloves were sanitized by spray them with 70% isopropanol and had been allowed to dry for 30 second before start the worked. There are three sizes of surgical gloves that small, medium and large.

4.1.2 Cell Irradiation

4.1.2.1 Siemens Primus Linear Accelerator (LINAC)

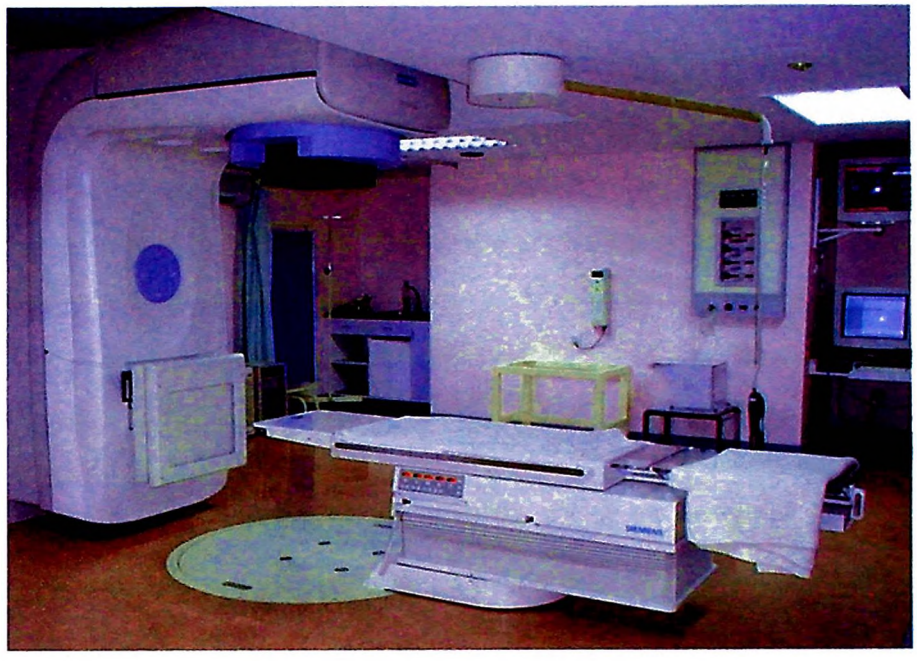

Figure 4.8: Linear accelerator (LINAC) used in HUSM

A linear accelerator or LINAC used in this research is Siemens Primus Linear Accelerator. This machine can produce different types of beam that are photon and electron beam with different energy of x-rays. LINAC is a machine that commonly used for external beam radiations treatments for cancer. It is used to treat all parts or organs of the body by delivering the high energy x-rays to the patient's tumor. The x-ray treatment will be planned in order to destroy the cancer cells while sparing the normal tissues.

4.1.2.2 Bolus

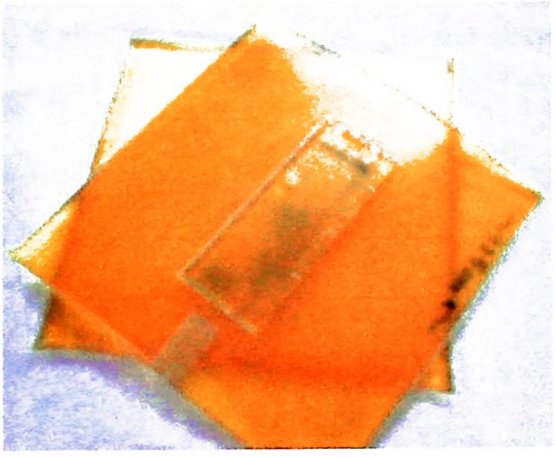

Figure 4.9: Bolus

Bolus is used to compensate missing tissue or irregular tissue shape and modifying dose at skin surface and at depth in radiotherapy. It is made up from a tissue equivalent material.

4.1.2.3 Solid water phantom

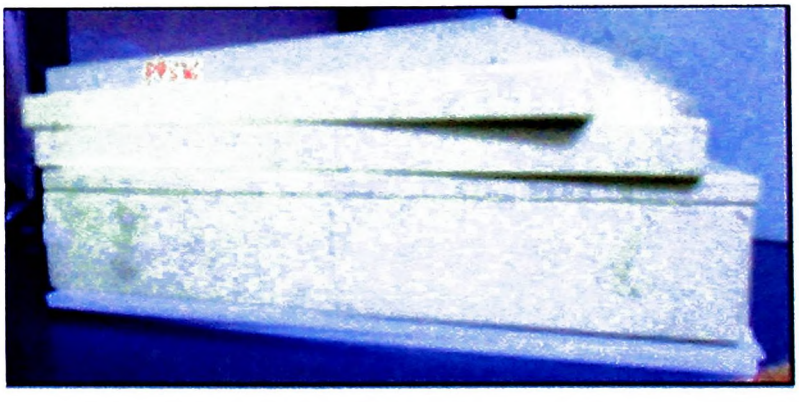

Figure 4.10: Solid water phantom

Solid water phantom is virtually the same as those in liquid water for the same depth and exposure duration. The common size of solid water phantom use is 30cm ×

30cm. However, solid water phantom also available in various sizes those are 20cm × 20cm and 40cm × 40cm. It also can be used for protons and electrons calibration and depth dose measurements. By using solid water phantom in this research, we do not need to use water tanks as replacement which is more convenient.

4.1.2.4 Electron applicator

10 cm × 10 cm^2 electron applicator was used to collimate the beam into 10 cm × 10 cm^2 in this research. Electron applicator was used only for electron beam energy set up.

4.1.3.1 Viability Assay of Cells

4.1.3.1 Hemocytometer

Hemocytometer was used to count the HeLa cells. This device originally designed for the counting of blood cells. A hemocytometer consists of a thick glass microscope slide with a grid of perpendicular lines etched in the middle that creates a chamber. The grid has specified dimensions so that the area covered by the lines is known and the depth of the chamber is also known. It is possible to count the number of cells in a specific volume of solution when the grid has specified dimensions.

4.1.3.2 Trypan blue

Figure 4.11: Trypan blue

Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. It is a ~960 Daltons molecule that is cell membrane impermeable and therefore only enters cells with compromised membranes. By using trypan blue, dead cell can be identified where the cells shows bluish color when trypan blue binds to intracellular