The Cell Survival of F10B16 Melanoma and 4T1 Breast Adenocarcinoma Irradiated to Gamma Radiation Using the MTT Assay Based on Two Different Calculation Methods

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ABSTRACT

Background: Various MTT assay methods are proposed to obtain the cell survival parameters.

Objective: Determining the survival curve characteristics of two cancerous cells of interest based on a common and a novel MTT assay method after exposing them to ionizing radiation.

Method: A common and a novel MTT assay method were used and compared for obtaining the F10B16 melanoma and 4T1 breast adenocarcinoma survivals after exposing them to ionizing radiation from a Co-60 machine. To obtain the survival parameters of the cells based on the common method, the cells were inoculated in 96-well plates. After irradiating the plates, the MTT assay was performed over the following days for a period of 8 days. Thereafter, the survival fraction was calculated from a simple equation for every day from which the best day was selected. To acquire the cells' survival parameters based on the novel method, extensive experiments were performed on a large number of samples. Then, the MTT assay was done in every day following various experimental treatments to acquire the exponential growth. Finally, the cells' survivals were determined by measuring the space between relevant growing curves.

Results: At low doses (<4Gy) the two MTT assay methods indicated the same results. However, at higher doses there were significant differences among the findings.

Conclusion: Both of the MTT methods indicated that the cells' responses are dependent on the dose levels used. Although the implementation of the common MTT assay method is simpler, the novel method seems to show more precise and reliable results at all levels of radiation doses.

Keywords

F10B16 melanoma, 4T1 breast adenocarcinoma, MTT assay, Cell survival fraction, Ionizing radiation, Growing curve

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Introduction

For determining the survival curve and its' relevant parameters for cancerous cells after exposing them to ionizing radiation, the basic common method used is the clonogenic assay [1-4]. However, this method has some limitations including the long time taken for colonies to form, the harm of possible pollution occurred in such a long period, the inability to measure the survival in the cells which do not grow to form colonies, and the errors inherent in counting the colonies

by the eye. Hence, alternative methods have been proposed such as the MTT assay. This assay can be performed with a large number of samples in short time using multi-well plates. Performing this assay is simple and cheap, and can also be carried out semi automatically with a microplate reader [1-4].

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay is one of the simple and available methods used for in-vitro measurement to obtain and compare the metabolic viability of the cell cultures under different treatment protocols. Viable cells reduce the yellow color of the MTT liquid to purple formazan crystals. The amount of the formazan crystals is proportional to the metabolic activity, and the crystals are produced by mitochondrial enzyme succinate dehydrogenase which is produced by live cells. Therefore, the number of live cells in the test sample is proportional with the amount of the colored formazan product determined spectrophotometrically after dissolving the formazan crystals in dimethyl sulphoxide [1-8]. It has been shown that the cells with poisoned mitochondria are able to produce the same amount of formazan when compared to the cells with normal mitochondria [9, 10].

The cells survival curve and its' relevant parameters can be calculated and determined mathematically from the MTT result using different formulas [1, 2, 4]. For studying the proliferation and determining the survival of the cancerous cells after exposing them to ionizing irradiation, the multiple MTT assay has been modified and used through different calculation methods proposed by different research groups [1, 2]. In this study, we have addressed, used, and compared the cell survival curve and relevant parameters as well as the growth characteristics of two cancerous cell lines of interest (F10B16 melanoma and 4T1 breast adenocarcinoma) using two different MTT based methods [1, 2]. The first method is an older but more common MTT based method and formula [2], while the second method is a novel different method and formula used and proposed recently in 2012 [1].

Methods

Cell lines and Culture conditions

Experiments were carried out on F10B16 (mice melanoma) and 4T1 (mice breast adenocarcinoma) cell lines. The cells were maintained in RPMI-1640 medium containing 10% heat-inactivated FBS (Gibco Laboratories, Cergy Pontoise, France), 500 µg/ml geneticin (G418), 300 µg/ml glutamine, 0.25 µg/ml fungizone, 100 µg/ml streptomycin, and 100 units/ml penicillin G.

The cell lines both were adherent and grew as monolayers at 37°C in a humidified 5% CO2 incubator. The cells were then harvested with 0.5 g/l trypsin (Gibco Laboratories) and 0.2 g/l EDTA (Gibco Laboratories) for 3 minutes. The concentration of the cells in the culture was adjusted to allow for exponential growth.

Irradiation procedure

After harvested with trypsin, the cells were counted and then plated in 96-well culture plates with almost 1000 cells/well. One day after the seeding of the cells, the cell cultures were irradiated to gamma rays from a Co-60 machine. All the culture plates including the controls were removed from the incubator at the same time and placed under the Co-60 source for the same period of time. However, the controls did not receive any ionizing radiation over this procedure since the source shutter has already been closed [11].

The ionizing radiation was delivered to the experimental groups as a single dose ranging from 0 to 10 Gy on a 25 cm × 25 cm field size. The source-half-depth distance was initially calculated to obtain a constant dose rate of 0.81 Gy/min. All irradiations were performed at a distance of 20 cm between the radiation source and the plates. 4 cm polystyrene block was used under the plates during the irradiation procedures to provide homogeneous

backscatter radiation.

To perform the calculation methods used for determining the cancerous cells survival curves and their relevant parameters, the MTT assay had to be done in multiple days. Hence, for each level of radiation doses (0, 2, 4, 6, 8, and 10 Gy) one plate was prepared for each day. We could have done this test for each column in the same plate, but avoided this procedure to circumvent the possible harm of pollution to other columns.

MTT Solution

The MTTs was dissolved in sterile phosphate-buffered saline at 5 mg/ml and stored in dark condition at 4 °C for a period lasting less than 3 weeks. After the final dilution with pre warmed sterile un-supplemented culture medium, the MTT solution was filtered through a 0.22µm filter.

MTT assay

For each cell line 14 wells of a 96-well plate was used for every experimental condition. The medium was renewed every two days to avoid possible medium product error. Since the cell lines were adhesive, their media could simply be renewed without making any damage to the cells. Separate plates were irradiated to different levels of doses of 0, 2, 4, 6, 8 and 10 Gy to obtain the relevant response points on the dose-response curve for every cell line. The MTT assay test was done once (or twice) every day to acquire the exponential growth of the cells. Based on a work done before [1], the MTT readings are proportional to the number of cells in-vitro at least in the exponential growth phase. To perform the MTT assay, the MTT solution at appropriate concentrations (10µl MTT solution in each 100µl media) was added to each well and the plates were then incubated at 37°C for 4 hours. Following the incubation, the remaining MTT solution was removed and 100λ of DMSO was added to each well to dissolve the formazan crystals. The plates were shaken for 5 minutes on a plate shaker to ensure adequate solubility. Absorbance readings of each well was performed at 540 nm (single wavelength) using a multi scan plate reader made in the UK. The control wells for absorbance readings contained no cells or medium, but similarly the DMSO was added to them. All the experiments were performed at least two times.

Survival calculation methods

To obtain the cells survival fraction, two different methods were used in this study. Based on one of the methods proposed earlier and used commonly [2] to obtain the cell parameters, the cells were inoculated in each well of the 96-well plates having a total volume of 200 µl/well and cultured for 18 hours at 37°C in a humidified 5% CO2 incubator for the adherence of the cells. After irradiating the plates, as mentioned above, the MTT assay was performed in the following days. It has been shown that there is a linear relationship between optical densities and cell numbers. The survival curve was drawn on a semi logarithmic scale of the survival fraction to dose. Then, the survival fraction was calculated from the following equation [2]:

The other method employed to determine the cell survival parameters of the cancerous

$$\text{Survival fraction} = \frac{\text{mean OD in test wells} - \text{mean OD in cell free wells}}{\text{mean OD in control wells} - \text{mean OD in cell free wells}} \qquad (1)$$

cells after exposing them to ionizing radiation was performed through studying the proliferation of the cells proposed recently in 2012 by Buch et al [1]. This kind of assay is done with a large number of samples in a short period of time using multi-well plates. Hence, to reach the aim of this study for examining the MTT assay as a replace of common clonogenic assay, the cells survival was also determined by the different method and mathematical formula proposed recently in that study [1] and compared with the former common method as explained above [2]. For this purpose, the exponential growth in the control a well as irradiated groups was provided from multiple MTT assay tests. The MTT assay was done once every day to acquire the exponential growth of the cells. Then, for calculating the cells survival through their proliferation, only the early exponential phase of the cells growth was used as indicated in the following equation:

$$Survival = 2^{-\frac{t_{delay}}{t_{doubling\ time}}} \qquad (2)$$

In which the tdoubling time is the time period required for a quantity of cells to double and the tdelay is the time period to reach specific absorption value of control versus irradiated cells.

Results

Primary results indicated that a number of 1000 cell/well is suitable and sufficient for the MTT assay test. Therefore, in the 14 wells of each 96-well plate, used for every cell line, about 1000 cells were put in 200µl culture medium and the remaining 4 wells were kept free of the cells to obtain the reference OD from their readings. All the other wells in each plate were filled with 200 µl PBS to maintain the humidity and uniformity of the plate during the irradiation treatment regimes made under the Co-60 source.

We did multiple MTT assay tests for several days after irradiation and calculated the relevant data and growing number of the mean OD of the test cells (including the cell lines of interest) from which the mean OD of the free cell wells had been subtracted. Then, the mean standard deviation of all the groups exposed to ionizing radiation was calculated.

At the first step, the doubling time of the two cancerous cell lines were obtained from the growing curve of the control as well as the treatment groups exposed to different levels of ionizing radiation. The growing curves of different groups are shown in Figures 1 and 2.

In addition, by using the growing curves, the

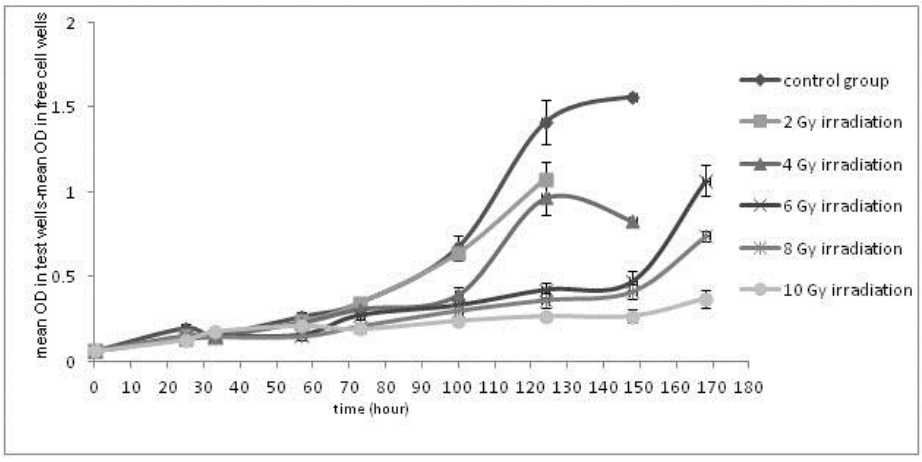

Figure 1: The growing curves determined for the F10b16 cell line in the control as well as different treatment groups exposed to different level of ionizing radiation.

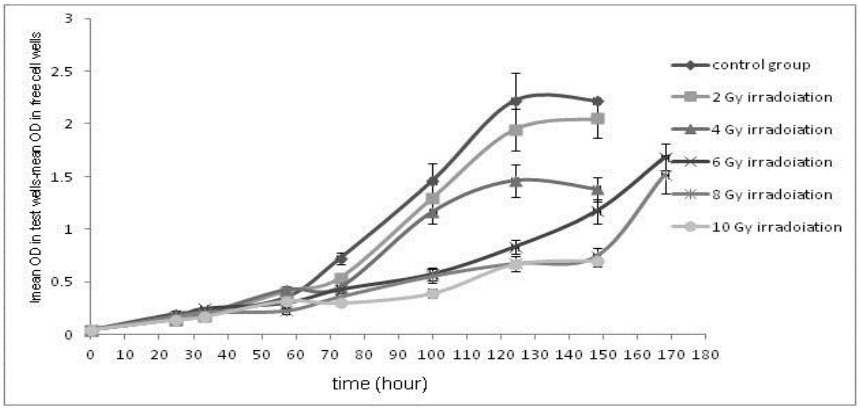

Figure 2: The growing curves determined for the 4T1 cell line in the control as well as different treatment groups exposed to different level of ionizing radiation.

time delay, doubling time, and survival fraction of the two cancerous cell lines were determined using the second method and mathematical formula proposed by Buch et al [1].

At the next step, the survival fraction of the cancerous cell lines was determined by using the first method and Equation 1 from the data obtained at various times after the irradiation of different control and treatment groups exposed to different levels of ionizing radiation doses [1, 2]. The survival fractions obtained by using this method are mentioned in Tables 1 and 2 for the F10b16 and 4T1 cell lines respectively.

Table 1: The survival fractions obtained using the first common MTT based method and formula (2) at various times (in hours) after irradiating the F10B16 cell line to different level of radiation doses.

DOSE(Gy)	SF 25h±SD	SF(33h)±SD	SF(57h)±SD	SF(73h)±SD	SF(100h)±SD	SF(124h)±SD	SF(148h)±SD
0	1	1	1	1	1	1	1
2	0.772±0.014	0.918±0.028	0.890±0.017	0.997±0.020	0.948±0.033	0.763±0.000	0.717±0.013
4	0.670±0.040	0.956±0.017	0.867±0.027	0.890±0.016	0.583±0.011	0.684±0.005	0.531±0.300
6	0.751±0.016	0.962±0.084	0.602±0.007	0.789±0.003	0.494±0.003	0.300±0.002	0.304±0.008
8	0.741±0.033	0.912±0.041	0.560±0.027	0.599±0.019	0.445±0.013	0.257±0.006	0.265±0.004
10	0.655±0.011	1.107±0.014	0.799±0.036	0.550±0.008	0.356±0.007	0.189±0.001	0.172±0.008

Table 2: The survival fractions obtained using the first method and formula (2) at various times (in hours) after irradiating the 4T1 cell line to different level of radiation doses.

DOSE(Gy)	SF 25h±SD	SF(33h)±SD	SF(57h)±SD	SF(73h)±SD	SF(100h)±SD	SF(124h)±SD	SF(148h)±SD
0	1	1	1	1	1	1	1
2	0.726±0.013	0.798±0.012	1.165±0.072	0.743±0.002	0.887±0.013	0.877±0.012	0.922±0.023
4	0.736±0.004	0.897±0.006	1.208±0.052	0.621±0.019	0.796±0.013	0.657±0.009	0.620±0.008
6	0.873±0.018	1.155±0.136	0.852±0.006	0.589±0.001	0.393±0.003	0.374±0.013	0.527±0.008
8	0.878±0.001	0.986±0.110	0.492±0.022	0.492±0.022	0.378±0.004	0.303±0.001	0.335±0.005
10	0.726±0.039	0.793±0.100	0.414±0.006	0.414±0.006	0.268±0.002	0.301±0.002	0.317±0.008

Survival fractions

The survival fractions obtained by the first method [2] showed that the cell numbers and OD at any fixed time after the irradiation does not produce significant different surviving fractions. If the survival is measured too early, during the exponential phase, or too late, when the control cultures reach to their confluence, the resulting survival fraction will be wrong.

In addition, a single day may not be sufficient to obtain survival fractions for the full range of ionizing radiation doses. Thus, in growth assay studies, it is recommended to obtain surviving fractions whenever treated cultures attain the exponential re-growth compared to the controls' growth rate. Hence, an experiment to define the time course of the growth of treated and control cultures is necessary in order to choose the optimum time at which the measurements must be done. The survival fraction curves obtained from the implementation of the two methods [1,2] for the two cancerous cell lines of interest (F10b16 and 4T1) are shown and compared with each other in Figures 3 and 4.

Conclusions

Cells survival following ionizing radiation is usually measured using clonogenic assay as the most common and reliable method. However, this method has some limitations including the long time taken for colonies to form followed by the harm of possible pollution, the inability to measure the survival in the cells which do not grow to form colonies, and the errors inherent in counting the colonies by the eye. The search for other more appropriate assays has been the subject of many research studies [1-7] over the years and the MTT assay is one of the alternative methods proposed for this purpose.

The advantages of the MTT assay include its' rapid semi automated reading, objective assessment, low cost, high reproducibility,

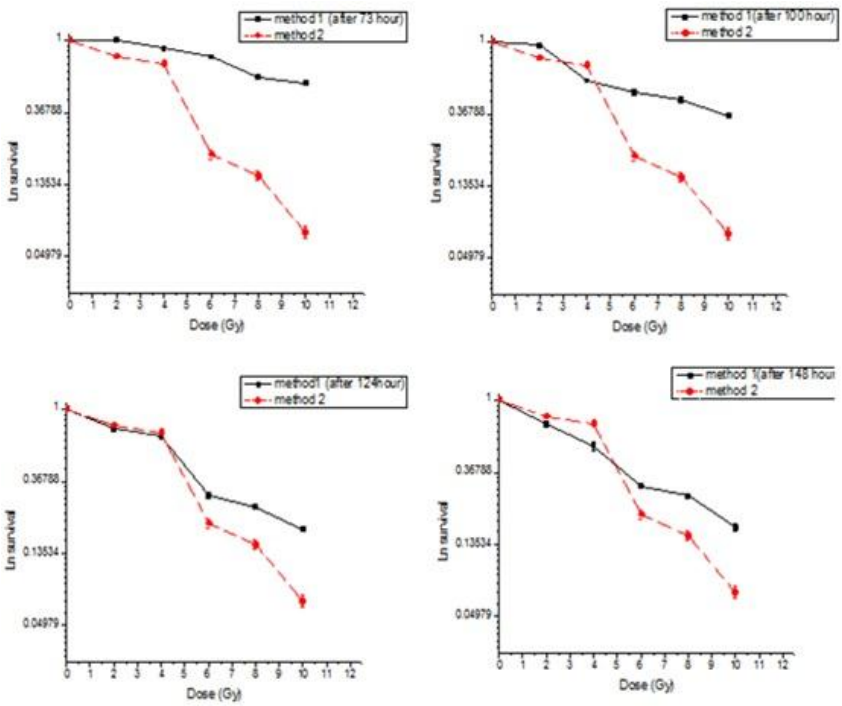

Figure 3: The survival fraction curves obtained from the implementation of the two MTT assay methods for the F10B16 cell line on a semi logarithmic scale.

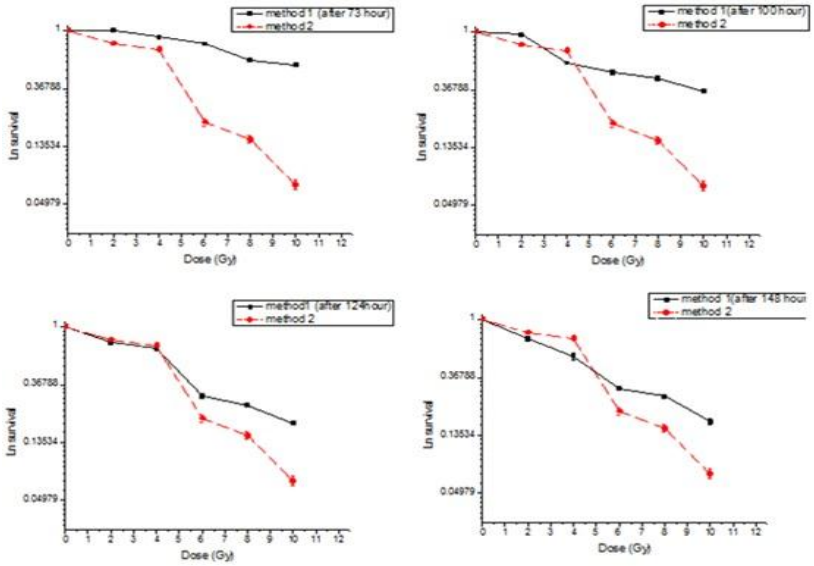

Figure 4: The survival fraction curves obtained from the implementation of the two MTT assay methods for the 4T1 cell line on a semi logarithmic scale.

low number of cells required, and the facility to quantify the cells grown in various conditions/forms such as suspensions, mono layers, spheroids, or clones.

On the other hand, different methods have been proposed in which the MTT is used instead of the clonogenic assay [1]. In this study, we investigated and compared two MTT based methods including an older but more common method [2, 5] used is most of the research works done before and another method suggested recently in 2012 by Buch et al [1].

With the first common method, the MTT assay test can be done on one specific day after irradiating the cells and their survival fraction can be obtained from only one set of data. This may bring about some problems since the best time for drawing the cells' survival curve has got to be found which is different for every cell line. Therefore, the application of this method is a difficult task and prone to errors at various irradiation conditions in which different level of doses may be used. But, the second method seems to be more reliable owing to its different approach making it independent from the conditions/limitations inherent in the first method.

As could be inferred from the results obtained from our experiments carried out on the two cell lines of interest (F10B16 and 4T1) leading to the survival fraction calculated at various times (73, 100, 124 and 148 hours) after the irradiation of the cells to different level of doses (2-8 Gy), some differences can be noticed between the two MTT based methods as depicted in Figures 3 and 4. For example, when the curves of these methods are compared, it can be noted that at low level of radiation doses (<4Gy) both of the curves don't show any significant differences and up to 124 hours after the irradiation they are nearly the same. However, at high level of radiation doses (>6 Gy) a large and significant difference between the two MTT based methods is observed at various time periods after irradiating the cell lines.

Discussion

Hence, it can be concluded that if the first common MTT assay based method [2, 5] is used; the test should be carried out up to about 124 hours after the irradiation of the cell lines for the radiation dose levels below 4Gy. But, when the second method is used, all the points drawn after various irradiation times as well

as different dose levels (even high doses) are reliable and also independent from the conditions/limitations of the first method. Hence, the second MTT assay based method [1] could be recommended to be used for drawing the survival curves of different cell lines instead of the clonogenic assay method, even though it seems to be more complicated and time consuming to implement and get all the required points compared to the other commonly used method.

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Conflict of Interests

None

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