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MTT assay instead of the clonogenic assay in measuring the response of cells to ionizing radiation

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Abstract

Although the clonogenic assay is the traditional method in determination of the response of cell lines to irradiation and in drawing the survival curves, but this method has some important defects such as the time taken to form clonies, inability to measure survival in cells which do not grow as clonies, and low sample throughput. Therefore, investigating other methods to situate this method is needed. One of the easy methods used widely in investigation of the effect of different treatments on cell lines, is the MTT assay. The MTT assay is a sensitive and accurate method and some researchers have used this method, considering its' several advantages such as relative low cost, short time to assess the samples and the possibility of acquiring the results semi-automatically. However, for this method, there are some restrictions/limitations and some important factors that should be discussed. In this paper, firstly, the standard procedure in using the clonogenic and MTT assay is described according to the standard protocols, then, different methods proposed in diverse researches in using MTT assay in calculating the cell survival instead of the clonogenic assay have been explained and compared.

Keywords: MTT assay, Clonogenic assay, Dose response, Survival curve

Introduction

The basic common method for determining the survival curve of cells after exposing them to ionizing radiation is the clonogenic assay (1-11). This method determines the ability of a cell line to proliferate indefinitely, in a way that retaining its reproductive ability to form a large colony or a clonies (12). Two different methods have been purposed to use this test to determine the cell survival curves which are called plating before and after irradiation (12,13). In both methods a long period of time is needed after irradiation to count the colonies and calculate the survival. Although, this test is the commune test in much investigations but has some disadvantages including: long time required to assess the samples, the possibility of polluting the samples over this long period, possibility to perform just on a low number of samples, prone to errors due to it's counting technique subjectively affected by the level of user expertise and skillfulness, and inability to be used on the cells which are not able to make clonies (13-18).

Hence, alternative methods have been proposed such as the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. In this method viable cells reduce the yellow color of the MTT liquid to purple formazan crystals (19). 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 (3,13-16,20-22).

The most important point in using MTT assay instead of the clonogenic assay is that, there is no difference between the morphologic end point caused by various factors affecting either the metabolic activity or the inactivation of the cells (15). Moerover, using this method has some advantages such as: a short time taken to assess the samples, possibility of assessing large number of samples simultaneously, possibility of acquiring the results objectively and semi-automatically using an instrument, high reproducibility, lower number of the cells required, possibility of assessing cells growth rate, possibility of using it on several types of the cell cultures (monolayer cells, spheroids, and clonies), and low cost (13-16,23,24). But in using this method truly some important points should be considered.

In this review, the correct method of using the clonogenic and MTT assays according to the previously published protocols is described. In addition, different methods proposed in other studies to use the MTT assay in determining the survival parameters instead of the clonogenic assay are compared.

Clonogenic assay

Basic idea

The basic idea in using the clonogenic assay method in order to plot the survival curves of cells is that, when

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■ **Implication for health policy/practice/research/ medical education**

Different methods have been proposed to calculate the cell survival after exposing them to ionizing radiation, such as; clonogenic and MTT assay. The basic common method for determining the survival curve of cells is the clonogenic assay. The MTT assay is a sensitive and accurate method and some researchers have used this method, considering its' several advantages such as: relative low cost, short time to assess the samples and the possibility of acquiring the results semi-automatically. In this review, the correct method of using the clonogenic and MTT assays according to the previously published protocols is described.

a cell is not dead, it has the capacity to divide and proliferate, therefore, can produce clonies or a large clony of cells (12,19).

Two different set up have offered in researches, including plating of cells before and after irradiation (12).

For plating after irradiation, cells should be grown in flasks and irradiated. In the next day, treated cells should harvest using trypsin, and then counted and a specific number of cells plated in petri dishes to form the colonies. The number of cells differs from 250 to 500 cells, according to their size and the rate of proliferation for each petri dish (3,13,25)

For plating before irradiation, cells from a stock culture are plated following appropriate dilutions according to the size and proliferation rate into 6-well-plates and are allowed to attach overnight. Irradiation is performed on the next day (12).

It should be noted that, in both of the methods, forming clonies are counted after about two weeks (3,13,25).

Material and method

To fixation and staining of clonies, after irradiation and seeding of the cells (if plating after irradiation method was used) is removed in both dishes and plates. Cells are rinsed with Phosphate Buffered Saline (PBS) and a mixture of 0.5% crystal violet in 50/50 methanol/water added for 30 min. Dishes are rinsed with water and are left for drying at room temperature (12,13). To count the clonies in each dish to obtain the plating efficiency, a dissecting scope to view the colonies under a magnified field is used. A cluster of blue-staining cells is considered a clony, if it comprises at least 25–50 cells (12).

Notifications

In counting the clonies some important notes should be considered such as:

1. It is important to keep the cut-off constant so that there are no variations introduced between the experiments.
2. If the cells remained single, not divided, or just had a few divisions and form small clonies, these cells would be scored as dead (12).
3. Because there is a long period of time between the seeding of the cells and counting, the cells should be kept carefully against different kind of pollutions (17,18).

Calculating the survival fraction

To calculate the survival fraction in all the protocols there is a formula, described as follow.

$$PE = \frac{\text{Number of clonies counted}}{\text{Number of cells}} \times 100 \qquad (1)$$

Where, PE is the plate efficiency. To calculate the fraction of the first generation of cells survival, all the PEs of the treated samples should be normalized to that of the control unirradiated plates. The surviving fraction (SF) is determined by dividing the PE of the treated cells by the PE of the controls, and then multiplying by 100 (12).

$$SE = \frac{\text{PE of treated sample}}{\text{PE of control}} \times 100 \qquad (2)$$

Survival curves for cells usually are presented with dose plotted on a linear scale and surviving fraction on a logarithmic scale (12,26,27).

MTT assay

Basic idea

MTT assay is the procedure of using yellow color of the MTT liquid to purple formazan crystals (19,28-30). 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 (3,4,13-15,23,25,31,32). 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 (3,25,33-35).

Material and method

To perform the MTT assay the 96-well plates are used for every experimental condition. The MTT-medium is prepared from 5 mg/1cc MTT in phosphate-buffered saline which is filtered and kept in the dark at 4 °C. The MTT solution at appropriate concentrations (10 µl MTT solution in each 100 µl media) should added to each well and the plates should then incubated at 37 °C for 4 hours. Following the incubation, the remaining MTT solution has to remove and 100 λ of DMSO added to each well to dissolve the formazan crystals. The plates have to shake for 5 minutes on a plate shaker to ensure adequate solubility. Absorbance readings of each well performed at a single suitable wavelength using a multi scan plate

reader (3,13, 15,16,36,37).

Notifications

Although MTT assay is simpler and more rapid than the clonogenic assay, but some notifications should be mentioned because this simple test may impress by some different factors, which are divided in two parts (3,15,38) that affect the spectrum of the produced formazan and amount of formazan produced per cell.

Therefore some important agents may help to have better results:

1. Using the proper mediums that could reduce the amount of light absorption in DMSO medium, which crystals will solved in.
2. Determining the best time to do the MTT test after irradiation of each cell line.
3. The spectrum of formazan, produced by cells, dissolved in DMSO is susceptible to pH. The peak shifts to a lower wavelength due to the addition of HC1 and color shift from purple-red to brown-red. Such spectrum shifts, will result in lower absorbance values, and can be overcame by adding a base such as NaOH to the solution.
4. A peak shift to a lower wavelength as observed after the addition of HC1 was also found with a formazan/DMSO which contained a residue of MTT-medium. To avoid this shift, it is essential to remove the MTT-medium as completely as possible before the addition of DMSO.
5. A spectrum shift may also occur as a result of the quality of the used DMSO. To remove this effect, the same DMSO medium should be used for all the samples, and, some free cell wells should be used which have tested with MTT and DMSO medium like the cell wells. Optical density (OD) of this wells have to subtract from the OD of the other wells (3,15,38).
6. When the number of cells increased and the culture medium went to acidic, culture medium of cells should be renewed every 2 or 3 days and also before adding the MTT medium.
7. The best incubating time for different cell lines is different and should be determined but almost the best time for much cells is 4 hour (15).

Calculating the survival fraction

Different methods have been proposed to calculate the cell survival by different researches (13,16). In this section these methods are explained and compared. In the research done by Kim et al. in 1993, the MTT test was used to determine the survival curve of hepatoma cells and claimed to be an appropriate method for determining the cell survival and dose-response curves due to the linear relationship between the cell numbers (<5000) and the MTT readings estimated by the following formula in which the test wells are those irradiated to ionizing radiation:

$$Survival\ fraction = \frac{\text{Mean OD in test wells} - \text{Mean OD in cell free wells}}{\text{Mean OD in control wells} - \text{Mean OD in cell free wells}} \quad (3)$$

A linear relationship between the OD and live cells has confirmed in this study. The authors have done the MTT test for a period of up to 9-11 days post irradiation for various cells and concluded that when about 5000 cells are used in each well, the 7th day is an appropriate day for determining the cell survival curve (16).

But in other research that has been done by Price et al. in 1990, it has emphasized that, there is a non-linear relationship between the MTT reading for higher numbers (>20000) (3). However, the important conclusion made regarding this matter/limitation of the MTT test was that when a large number of cells (>20000) are used in the wells, a calibration curve is required to be calculated, but there will be no need for such calibration if the cell numbers is lower due to the linear relationship existed between the MTT readings and the cell numbers. This claim has also been proved by other researchers (31).

In addition, it has also pointed out that comparing simply the cell numbers of experimental groups at a given day (post irradiation) with that of the control group did not correctly provide the cell survival (3,39,40). This has been attributed to the fact that radiation causes a delay in the re-growth of the cells. As in the clonogenic test the cells ability to make even small clonies is taken into account for calculating the growth rate, it is recommended that for the MTT test the survival rate of the irradiated cells should be measured continuously for several days post irradiation in order to determine the precise period in which the cells get back to their exponential growth phase and become similar to that of the controls. Thereafter, the cell survival should be calculated from the displacement of the irradiated cell growth from that of the controls when their growth curves become parallel to that of the controls. They have done this approach for the RT112 cell line and claimed that the relevant cell survival determined from the MTT assay had been in a good agreement with that of their clonogenic test (14).

In other investigation by Sieuwerts et al. in 1995, the application of the MTT assay for determining cells growth characteristics and survivals has been investigated in which, apart from the source of errors, the relationship between the samples cell numbers and ODs has been discussed. These authors have also proved that when the number of cells is low, there will be a linear relationship between the cell numbers and relevant ODs measured in MTT test (15).

In the other research by Buch et al. in 2012, the authors have determined and compared the cell survival curves using both of the MTT and clonogenic tests in an attempt to replace the clonogenic assay test with the MTT. They

have pointed out that, in many studies that only the MTT test has been used, for determining cell survivals, some important characteristics and parameters such as: the cell doubling time, and the delay induced in irradiated cells and their growth behavior have been ignored. Therefore, they have proposed an appropriate method for determining the survival curves in which these confounding parameters are taken into account. For this purpose, they have used consequential MTT tests with several samples in which various numbers of cells have been used in each well and the numbers of clones have been counted for a period of up to 9 days post irradiation.

It was indicated that a more precise equation that has proposed for deriving the cell survival curve from the MTT test has been led to the results in good agreement with that of the clonogenic assay, especially with the clonogenic test with plating before irradiation (3,4,14,41-43).

As could be seen from the Figure 1 (13), the growth curves are drawn for the control and irradiated groups.

Then, the irradiated groups' delay time has been calculated based on the time differences of their growth curve with that of the controls at the exponential region of the curves and finally their survival fraction has been defined from the following equation:

$$SF = 2^{-(\frac{t_{delay}}{t_{doubling\ time}})} \quad (4)$$

In which the t_{delay} is the amount of the time required to pass in the exponential region of the irradiated cells to reach the same survival rate of the control group and $t_{doubling\ time}$ is the time period in which the number of the cells becomes double.

Following the above studies an investigation has done by our group in 2013 (44), to compare the formulas used in previous studies. The multiple MTT assay tests performed for several days after irradiation and the relevant data calculated. The doubling time of the two cancerous cell lines obtained from the growing curve of the control as well as the treatment groups exposed to different levels of ionizing radiation. In addition, by using the growing curves, the time delay, doubling time, and survival fraction of the two cancerous cell lines determined, using the methods and mathematical formulas proposed by Kim et al. and Buch et al. (13,16). The results showed that, if the first common MTT assay based method of Kim et al 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 4 Gy. 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 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 (44).

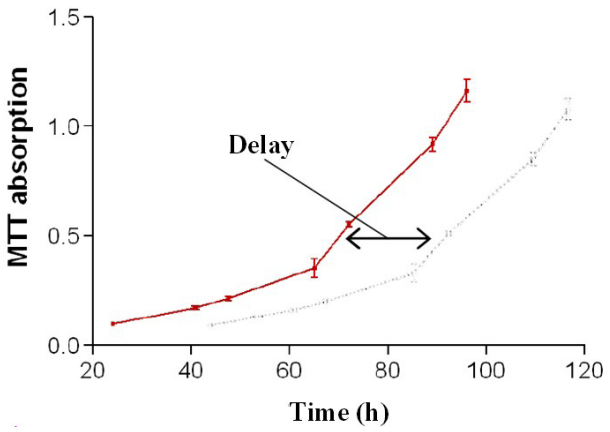

Figure 1. The growth curves for the control and irradiated groups (13).

Conclusions

In this paper, different methods that have proposed to calculate the cell survival by different researches are explained. The MTT assay is a sensitive and accurate method and some researchers have used this method, considering its' several advantages such as relative low cost, short time to assess the samples and the possibility of acquiring the results semi-automatically. However, for this method, there are some restrictions/limitations and some important factors that should be considered further.

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Author's contribution

All authors wrote the manuscript equally.

Ethical considerations

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Conflict of interests

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Compliance with Ethics Requirements

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