Dose-response modeling using MTT assay: a short review

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Abstract: MTT assay is widely used to determine the response of cells to ionizing radiation. This method has 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. This paper is focused to discuss a standard procedure in using MTT assay for calculating the cell survival after irradiation.
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1. Introduction

MTT assay is widely used to determine the survival curve of cells to ionizing radiation (1-6). In this method a short period of time is needed after irradiation to count the colonies and calculate the survival (6-13).

In 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay, viable cells reduce the yellow color of the MTT liquid to purple formazan crystals (13-19). 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 clones), and low cost (13-16, 23, 24). But in using this method truly some important points should be considered. In this paper, the standard method of using the MTT assays is described.

1.1. MTT assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (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).

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).

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 determine but almost the best time for much cells is 4 hour (15).

1.2. 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:

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

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 clones 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 (40-47). 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 (47-51). 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 (51-55). 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).

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:

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

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 (55-61), 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 (61-64). 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 (64-71). 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, 71-78).

2. Conclusions

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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