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Multiple MTS Assay as the Alternative Method to Determine Survival Fraction of the Irradiated HT-29 Colon Cancer Cells

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ABSTRACT

A multiple colorimetric assay has been introduced to evaluate the proliferation and determination of survival fraction (SF) of irradiated cells. The estimation of SF based on the cell-growth curve information is the major advantage of this assay. In this study, the utility of multiple-MTS assay for the SF estimation of irradiated HT-29 colon cancer cells, which were plated before irradiation, was evaluated. The SF of HT-29 colon cancer cells under irradiation with 9 MV photon was estimated using multiple-MTS assay and colony assay. Finally, the correlation between two assays was evaluated. Results showed that there are no significant differences between the SF obtained by two assays at different radiation doses ($P > 0.05$), and the survival curves have quite similar trends. In conclusion, multiple MTS-assay can be a reliable method to determine the SF of irradiated colon cancer cells that plated before irradiation.

Key words: *Colon cancer cells, colony assay, multiple-MTS assay, plating before irradiation*

INTRODUCTION

Colorectal cancer is the third most common cancer in the world. However, despite all the recent developments in cancer therapy, colon cancer recurrence (50%) has been remained as a major problem, and it is necessary to develop some new more effective approaches for achieving a certain level of cell mortality.[1-3] Therefore, introduction of a rapid and reliable method to estimate survival fraction (SF) is essential to investigate the colon cancer cells sensitivity to different treatments.

It has been shown that clonogenic assay or colony formation assay is a perfect method for SF estimation of irradiated cell. It relies on the ability of a single cell to grow into a colony that consists of at least 50 cells. However, this method has some disadvantages such as long sample preparation time, labor-intensive, contamination risk, and errors prone. In addition, this assay is not suitable for all kinds of cells because some cell types are unable to form colonies.[4-6] Therefore, alternative colorimetric methods have been proposed to determine the cell response to irradiation based on assessing cell metabolic activity. Under defined condition in viable cells, NAD (P) H-dependent cellular oxidoreductase enzymes may be capable of converting tetrazolium into colored formazan. The amount of the formazan is proportional to the metabolic activity of mitochondrial enzymes which are produced by live cells. Using a spectrophotometer, the quantity of produced formazan can be measured by recording the changes in a specific absorbance wavelength. A variety of tetrazolium compounds have been used to detect alive cells by the colorimetric assay. The most commonly used compounds include MTT, MTS, XTT, and WST-1.[4,7-10]

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction produces a formazan product that is insoluble. Hence, a proper method to solubilize the

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formazan product is needed. The MTS (3-(4,5-dimethylthiazol -2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetraz olium) generate formazan products that are directly soluble in cell culture medium and often described as a "one-step" MTT assay. This properties make the MTS assay more convenient.[5]

The colorimetric assay is a single-point assay that run after a defined time following irradiation. In this method, the SF can be obtained from only one set of data, which may be the cause for the loss of some important information on the growth performance, and therefore the SF may be misestimated. It seems multiple-colorimetric assay is an appropriate method to cope with this problem.

Recently, Buch *et al*.[11] described a new multiple-colorimetric assay to estimate the SF of irradiated cells. Since in this method, the assay will be done once every day and the used tetrazolium compound is MTT, this new method is called "multiple MTT-assay." In multiple-MTT assay, the cell growth data are collected daily to draw the cell growth-curve. The SF will be estimated by measuring the time difference between the cell growth of the control and the treated groups to reach a certain value. Therefore, in comparison with the colony assay, multiple-MTT assay provides an opportunity to measure the more precise SF for a larger sample in less time and with less subjective errors. Regardless of the advantages of this method, the most important disadvantage of the multiple-MTT assay is the probability of overestimation of SF for high radiation doses in plating before irradiation-setup.

Routinely, plating before irradiation-setup is used for a rapid screening of cells' sensitivity to different treatments. Therefore, to evaluate the effects of new therapeutic approaches, survival assays should be done by plating before irradiation-setup.[12,13] Based on literature,[11] to replace the colony assay with, using multiple MTT-assay plating before irradiation-setup, correlations of multiple MTT and clonogenic assay at two or three radiation doses should be verified for each cell type.

In the present study, regarding the importance of developing a rapid and reliable method to investigate the colon cancer cells' sensitivity to the different treatments, the reliability of multiple-colorimetric assay for HT-29 cells in the case that is plated before irradiation was examined using multiple-MTS assay.

MATERIALS AND METHODS

Cell Culture

HT-29 cell line was purchased from Pasteur Institute (Tehran, Iran). All cells were maintained in RPMI medium 1640 (Gibco-Invitrogen) supplied by 10% fetal bovine serum (Gibco-Invitrogen) and 1% antibiotic mixture containing penicillin (Sigma-Aldrich) and streptomycin (Sigma-Aldrich). The cells were stored at humidified atmosphere at 37°C with 5% CO_2. The medium of cells was changed approximately every 2 days. The cells were split with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid when they reached 80% confluency and sub-cultured for further passages.

Plating Before Irradiation

Single-cell suspension was seeded in a 6-well-tissue culture for colony assay (1000 cell/well) and in 96-well tissue culture plates (4000 cell/well) for multiple MTS-assay and was stored at humidified atmosphere at 37°C with 5% CO_2 overnight.

Irradiation Procedure

Megavoltage X-ray irradiation was performed using a clinical accelerator (Neptun 10 PC, located in radiotherapy section of Seyed Alshohada Hospital, Isfahan, Iran) at source-to-surface distance = 100 cm, and 20 × 20 cm^2 field size, which was described previously.[9] Briefly, 2 cm of a Plexiglass sheet (water equivalent) was placed on the top of the plate. The plate was placed in a phantom made of Plexiglass with a sized cavity of 12.5 × 8.5 × 1.5 cm^3 at the center [Figure 1]. Cells were irradiated with photon of 9 MV at a total dose of 2, 4, 6, and 8 Gy with a dose rate of 300 cGy min^{-1}. *In vivo* radiation diode dosimetry measurements were done for the beam calibration, and the variation within a field was smaller than 2% for each well. After irradiation, cells were incubated at 37°C, 5% CO_2.

Multiple MTS-assay

After irradiation, MTS-assay was performed once every day. Cell viability was investigated using the Cell Titer 96 AQueous One Solution Reagent (Promega, USA). Briefly, RPMI (100 µl) was supplemented with MTS solution 20 µl/well, incubated for 2 h, and then the absorbance was recorded at 490 nm with a 96-well plate reader (Biorad).

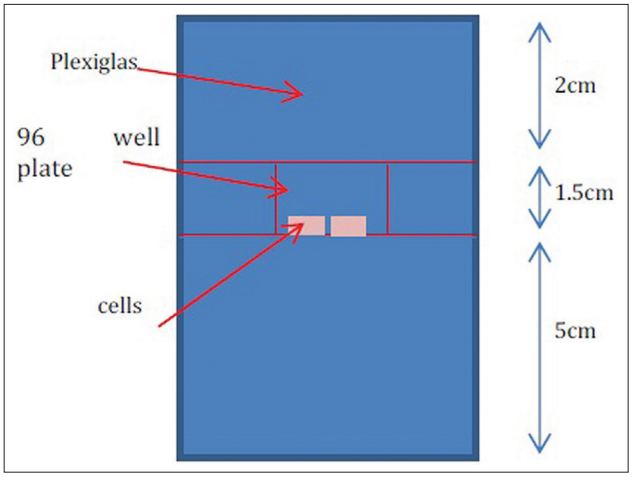

Figure 1: Schematic cross-section of the cell irradiation phantom

Colony assay

Fourteen days after irradiation, the medium above the cells was removed and rinsed with phosphate-buffered saline. Fixation and staining of colony were done carefully with 4% paraformaldehyde solution, hematoxylin and eosin stain, respectively. Colonies exceeding 50 cells were counted with Image Master 2D platinum 6.0 (General Electric Healthcare Life Sciences, USA).

Statistical Analysis

All values were expressed as means ± standard deviations. Differences $P < 0.05$ were considered statistically significant. All the experiments were performed in triplicate and repeated at least 3 times.

RESULTS

Multiple MTS-assay

As mentioned earlier, MTS assay was performed once every day to obtain exponential growth of cells. To calculate the proliferation survival, only the early exponential phase of cell growth is used, according to Eq. 1.[11]

$$\text{Survival} = 2^{\frac{-t_{\text{delay}}}{t_{\text{doubling-time}}}} \quad (1)$$

Where $t_{\text{doubling-time}}$ is the period of time required for duplication of a quantity of cells and t_{delay} is the time period to reach specific absorption value of control versus irradiated cell.[11]

Figure 2 shows the cell growth curves of the control (nonirradiated) and the irradiated groups. In addition, the delay times in the cell growth of the irradiated cells versus control cells were shown in Figure 2. The survival results based on multiple-MTS assay are assessed [Table 1].

Colony assay

After 14 days, colonies were stained and counted. The SF was calculated using Eq. 2,[13] and the results are shown in Table 1.

$$\text{PE} = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times 100$$

$$\text{SF} = \frac{\text{Number of colonies formed after irradiation}}{\text{Number of cells seeded} \times \text{PE}} \quad (2)$$

In Figure 3a, survival curves of HT-29 cells fitted using the LQ-model[14] for clonogenic and multiple-MTS assay are shown. As seen in Figure 3a, clonogenic and multiple MTS assay show very similar curve progression. A good correlation between the two assays was obtained with $R^2 = 0.95$ [Figure 3b].

DISCUSSION

The relationship between the radiation dose and cell SF can be derived using the cell survival curve. In the radiation biology research, there are two ways to explore cell behavior. One of them is "plating before irradiation" which is often used to investigate the sensitivity of different treatments. The other one is "plating cells after irradiation" which is used to determine potential lethal damage or sublethal damage repairs.[12,13]

The method described by Buch *et al*. introduced multiple-colorimetric assay based on MTT dye and

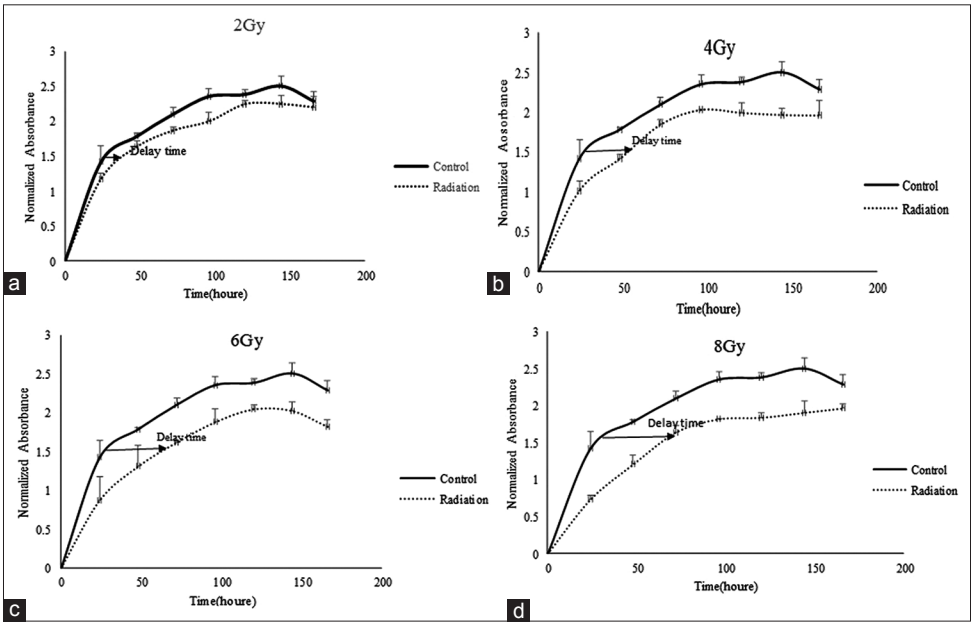

Figure 2: The growth curve of cells based on multiple-MTS assay and delay in cell growth of irradiated cells (dash line: a [2 Gy], b [4 Gy], c [6 Gy], d [8 Gy]) versus control cells (solid line: 0 Gy)

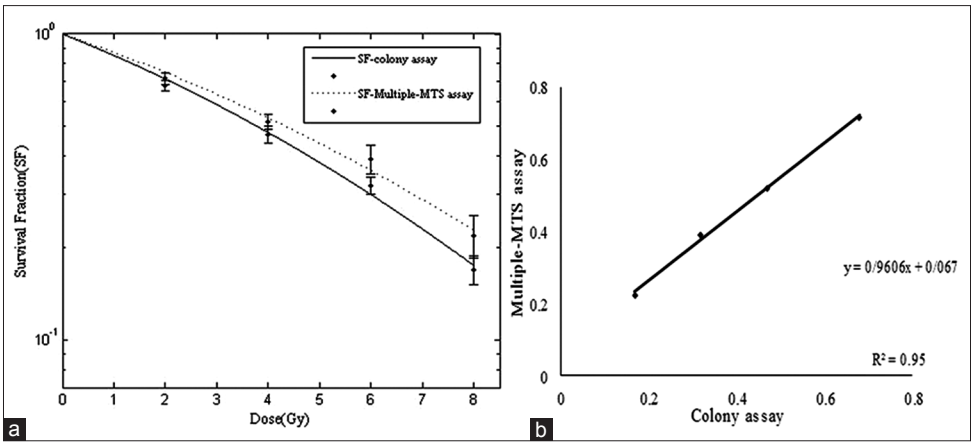

Figure 3: (a) Survival curve obtained from the implementation of colonogic and multiple MTS-assay. (b) Correlation of both mentioned methods

Table 1: Survival of irradiated cells based on multiple-MTS assay: (4000 cells) ($n=6$ for each experiment) and colony assay: (1000 cells) ($n=3$ for each experiment)

Dose (Gy)	SF (multiple-MTS assay)	SF (colony assay)	*P*
2	0.71±0.03	0.68±0.026	0.33
4	0.52±0.03	0.47±0.01	0.10
6	0.39±0.04	0.32±0.002	0.17

SF: Survival fraction

multiple-MTT assay for the evaluation of irradiated cell behavior in both plating of cells before or after irradiation setups.[11] They have shown that in plating after irradiation-setup, the correlation between multiple-colorimetric and colony assay for different cell lines is very good (R^2 above 96%). Alive cells with a low metabolic activity and no cell proliferation may lead to overestimate the SF. In the plated after irradiation cases, these cells are removed by washing and trypsinization. Whereas, for the plating before irradiation-setup, depending on the cell type, these low cell activity may cause some error in SF estimation, especially at a high radiation dose. For example, a good correlation has been shown between two assays for A-549 and F-98 cell lines (99% and 98%, respectively) in plating before irradiation setup, but this correlation decreased for LN-229 cells ($R^2 = 0.72$). Therefore, they concluded that the possibility of replacing of multiple-colorimetric assay versus colony assay should be verified for each cell line at least in two or three radiation doses.

A proper medium must be used to reduce the amount of light absorption in dimethyl sulfoxide (DMSO) medium, in which crystals will be solved. In addition, in each study, the optimum time for the MTT test after irradiation of each cell line is of outmost important.[15] Moreover, the spectrum of formazan, produced by cells, dissolved in DMSO is susceptible to pH. This causes peak shifts in the spectrum. To avoid this shift, it is essential to remove the MTT-medium as completely as possible before the addition of DMSO.[16,17] Furthermore, 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.[17-19] Finally, the best incubating time for different cell lines is different and should be determined, but almost the best time for such cells, used in this study, is 4 h.[20]

In this study, we used MTS dye that is more convenient and precise instead of MTT to simplify our method, and we named it as multiple-MTS assay. Our results that at different radiation doses, there is no significant difference between multiple-MTS assay and the colony assay in the estimation of the HT-29 SF that plated before irradiation ($P > 0.05$). The survival curves of irradiated HT-29 colon cancer cells for both assays showed similar behavior, so the correlation of two assays was acceptable ($R^2 = 0.95$). These findings are in agreement with results which have been reported recently for adenocarcinomic human alveolar basal epithelial cells and the rat glioma tumor cells.[11]

Consequently, it seems that the multiple MTS-assay may be a suitable alternative of colony assay to estimate SF of irradiated colon cancer cells for the plating before irradiation setup.

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Conflicts of Interest

There are no conflicts of interest.

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