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Evaluation of MTT and Trypan Blue assays for radiation-induced cell viability test in HepG2 cells

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► ***Original article***

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ABSTRACT

Background: Cell viability is an important factor in radiation therapy and thus is a method to quantify the effect of the therapy. ***Materials and Methods***: The viability of human hepatoma (HepG2) cells exposed to radiation was evaluated by both the MTT and Trypan blue assays. The cells were seeded on 96 well-plates at a density of 1 x 10^4 cells/well, incubated overnight, and irradiated with 1-100 Gy. ***Results:*** The cell viability was decreased in a dose- and time- dependent manner when using the Trypan blue assay, but no significant changes in the response to dose could be detected using the MTT assay. It indicated that the MTT assay was not efficient at a cell density of 1 x 10^4 cells/well on 96 well-plates to determine cell viability. Subsequently, the relationship between cell viability and lower cell density (1 x 10^3, 3 x 10^3, and 5 x 10^3 cells/well) was investigated. A cell density of 1 x 10^3 was found to be the most effective when using the MTT assay. Results show that the cell density is most important when using the MTT assay in 96 well-plates to follow in radiation effects. Furthermore, the radiation-induced cell viability dependent on cell density was confirmed by using the traditional Clonogenic assay. ***Conclusion:*** Our results suggest that the MTT and Trypan blue assays are rapid methods to detect radiation-induced cell viability of HepG2 cells in about 3 days as compared with 14 days of assay time in the Clonogenic assay. To obtain accurate cell viability measures using both rapid assays, an incubation time of at least 3 days is needed after irradiation.

Keywords: *Cell viability, clonogenic assay, HepG2 cell, MTT, radiation, trypan blue.*

INTRODUCTION

Radiation therapy is a very effective treatment modality for cancer [1, 2]. Human hepatocellular carcinoma (HCC) is a liver cancer and the fifth most common cancer in the world [3]. Human hepatoma (HepG2) cells are used as a HCC model *in-vitro* [4]. Radiation can lead to cell death [5, 6]. In cancer treatment, cell viability is a basic and important parameter for predicting radio-sensitivity in the treatment of human cancer [7, 8]. Radiation-induced cell viability is also a significant work for biological research such as DNA repair, cell cycle, and apoptosis [9, 10]. To date, the clonogenic assay has been extensively used for measurement of cell viability in radiation study [11-16]. However, the conventional clonogenic assay remains unsatisfactory. It is a colony formation assay which is labor-intensive and time-consuming (incubation time; 1-2 weeks). Therefore it is necessary to look over a rapid and easy assay for determination of radiation-induced cell viability.

Both the MTT and Trypan blue assays are routine and convenient methods for determination of cell viability [17, 18]. The MTT assay is a colorimetric assay, which is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals only by viable cells. Usually, it is performed in 96 well-plates and measured the absorbance using the micro-plates reader. The Trypan blue assay is a dye exclusion

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staining assay, which is based on uptake of trypan blue dye by dead cells due to loss of their membrane integrity, so the dead cells appear darker than the viable cells. It is measured by using a hematocytometer and a microscope or cell counting instruments.

The aim of this study was to compare the MTT and Trypan blue assays for radiation-induced cell viability in cultured HepG2 cells on 96 well-plates. Also, we determined the relationship between cell viability and cell density after irradiation and confirmed the radiation-induced cell viability according to cell density by using the Clonogenic assay.

MATERIALS AND METHODS

Materials

Trypan blue reagent, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Penicillin, and other cell culture reagents were purchased from Gibco BRL (France). Tetrazolium (MTT) was purchased from Roche (Mannheim, Germany). Crystal violet was purchased from YD diagnostics (Gyeonggi, Korea). All other reagents were obtained from analytic grade.

Cell Culture

HepG2 cells were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin (100 U/ml) at 37 ^{o}C in 5% CO_2. The cells were harvested following trypsinization (0.025% trypsin and 0.02% EDTA) and washed twice with phosphate buffered saline (PBS). When the cell density reached approximately 80% confluence, the cells were subcultured. The cell viability was determined using the Trypan blue, MTT, and clonogenic assay. Cells (1000, 3000, 5000, and 10000 cells/well) were seeded in 96 well-plates, incubated overnight, and irradiated with 1-100 Gy. Then, the cells incubated for 1, 2 and 3 days, respectively. These samples were used for different cell viability assays (MTT, Trypan blue, and clonogenic assay).

Typan blue assay

The Trypan blue assay was measured by previously described [17]. One to three days after irradiation, cells were detached by trypsinization and the number of viable cells was counted using a Trypan blue stain reagent. The viability of the control (untreated cells) was regarded as 100%.

MTT assay

The MTT assay was measured by previously described [18]. One to three days after irradiation, cells were treated with MTT reagent. The absorbance at 570 nm was measured using a microplate reader (Mutiskan EX, Thermo Lab systems). The viability of control (untreated cells) was regarded as 100%.

Clonogenic assay

The clonogenic assay was performed according to the procedures previously described [19]. After irradiation, cells were incubated for 1, 2, and 3 days, respectively. Then, cells were trypsinized, counted, and seeded in triplicated in 100-mm dishes (100 and 500 cells per dish) and incubated for 14 days to allow for colony growth. After 14 days, colonies are fixed with 70% ethanol, stained with crystal violet (0.3%) and counted using a counter. The survival (%) was calculated as (number of colonies/ number of cells plated)/(number of colonies for corresponding sham-irradiated control/number of cells plated)×100.

Irradiation

Cells were irradiated with gamma radiation from a ^{60}Co gamma irradiator (7.4 PBq od capacity; AECL, Canada) at Korea Atomic Energy Research Institute. The radiation dose was 1, 5, 10, 50, and 100 Gy, and the dose rate was 0.05, 0.25, 0.5, 2.5 and 5 Gy/min, respectively.

Statistical analysis

Data are expressed as mean ± SD from three replicates at least. Statistical analyses were performed using Sigma Stat software. The significance testing was performed using student's t-test (*: P<0.05 versus control).

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RESULTS

Radiation-induced cell viability in HepG2 cells using Trypan blue and MTT assay

To compare the cell viability effect of radiation on HepG2 cells, we used MTT and Trypan blue assays (figure 1). Cell viability was determined over 3 days using the two assays. When cells were seeded in 96 well-plates at an initial cell density of 1×10^4 cells/well, the viability was decreased in a dose-dependent manner by using the Trypan blue assay (figure 1A). The cell viability was about 50% for 3 days after 5 Gy irradiation in the Trypan blue assay. However, the viability value of the MTT assay displayed no significant changes (figure 1B). Even after radiation of up to 100 Gy, the decrease of viability was not observed.

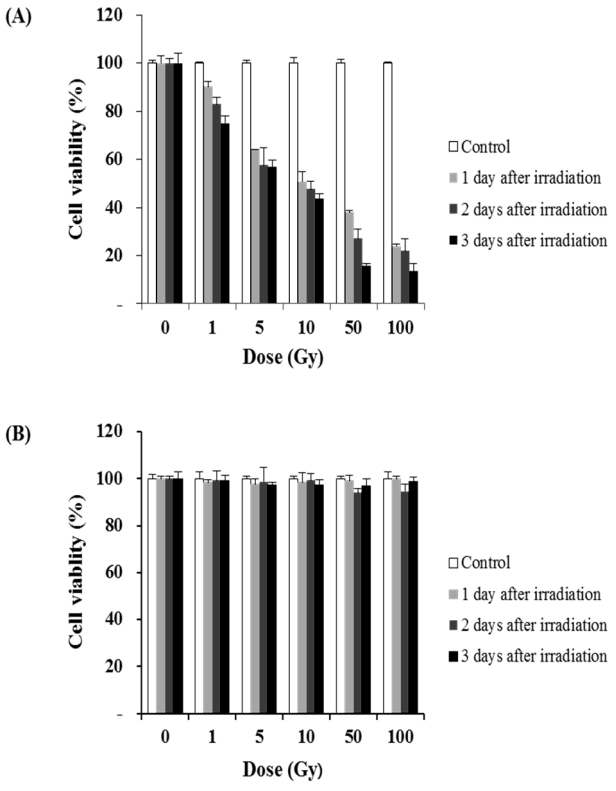


Figure 1. Radiation-induced cell viability in HepG2 cells by using the Trypan blue assay (A) and MTT assay (B). Cells were plated on 96 well plates (1×10^4 cells/well) for 1 day and then irradiated with irradiation with 0 – 100 Gy. After incubation for 1, 2 and 3 days, cell viability was measured by both assays, respectively. Data are mean ± SD of triplicate determinations.

To study why the MTT assay was not effective to cell viability, we examined the relationship between cell viability and cell density (1×10^3, 3×10^3, 5×10^3 and 1×10^4 cells/well) by using the MTT assay (figure 2A). When the cell density was 1×10^3 cells/well, the viability was decreased by about 50% for 3 days after 5 Gy irradiation. In the cell density of 1×10^3 cells/well, the cell viability measured by the MTT assay was decreased in a dose-and time-dependent manner (figure 2B). Also, we tested the Tyrpan blue assay at the same cell density. In the Trypan blue assay, the viability was measured as a dose- and time- dependent pattern when the cell density was 1×10^3 cells/well (data not shown).

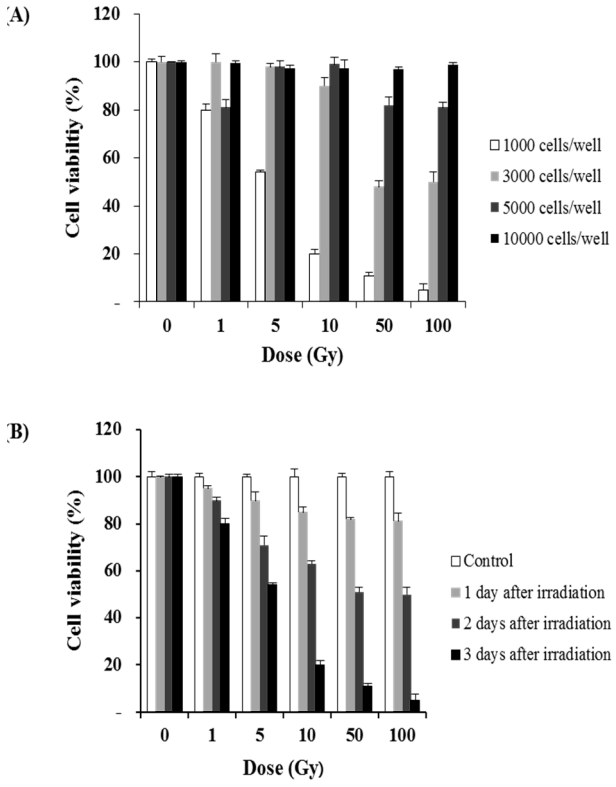


Figure 2. The relationship between cell viability and cell density (1×10^3, 3×10^3, 5×10^3 and 1×10^4 cells/well) 3 days after irradiation (A) and time-course of cell viability at cell density (1×10^3 cells/well) one to three days after irradiation (B). Cells were plated on 96 well plates (indicated cell density) for 1day and then irradiated with irradiation with 0 – 100 Gy. After incubation for indicated days, cell viability was measured by the MTT assay, respectively. Data are mean±SD of triplicate determinations.

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Radiation-induced cell survival in HepG2 cells using clonogenic assay

To confirm that radiation induces cell death in HepG2 cells, we performed the clongenic assay. When the cell density was 1 × 10^4 cells/well, radiation resulted in a decrease of cell survival (%) in a dose-dependent manner (figure 3). After 5 Gy irradiation, there was about 50% of cell survival. However, there was no colony formation at 50 and 100 Gy. Regardless of the initial cell density (1 × 10^3- 1 × 10^4 cells/well) and the incubation time (1-3 days) after irradiation, 5 Gy radiation induced about 50% of cell death (data not shown).

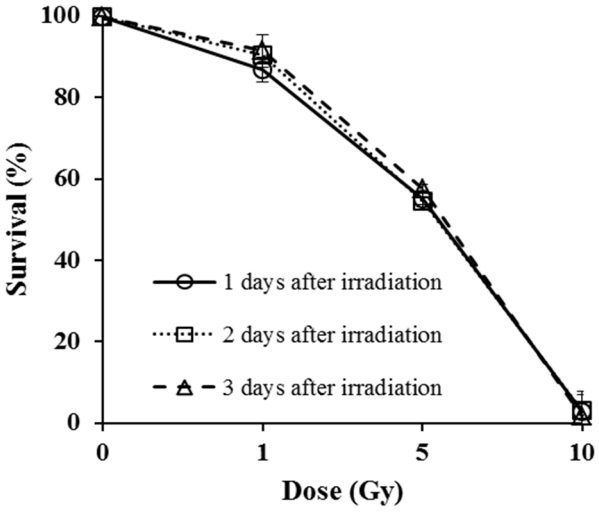


Figure 3. Radiation-induced cell survival in HepG2 cells. Cells were plated on 96 well plates (1 × 10^4 cells/well) for 1 day and then irradiated with 0 – 100 Gy. After incubation for 1, 2 and 3 days, the clonogneic assay was carried out according to the procedures described in Materials and Methods. Data are mean ± SD of triplicate determinations.

DISCUSSION

Radiation has extensively been used as a tool of cancer treatment [1, 2]. The exposure of cancer cells to radiation can lead to cell death such as apoptosis or necrosis [20]. It was confirmed that HepG2 cells after irradiation resulted in a dose-dependent viability loss by using the MTT and Trypan blue assays in this study.

There are various assays for cell viability such as the Trypan blue [17], MTT [18], XTT [21] and clonogenic assay [19]. Among these assays, the clonogenic assay has exclusively been used for determination of cell survival in radiation study. However, there has been little report about the evaluation of MTT and Trypan blue assay on HepG2 cell viability after irradiation. In this study, it was found that the MTT, Trypan blue and clonogenic assay gave similar results for radiation-induced cell viability under different condition (cell density and assay time). After irradiation, the incubation time was 3 days for the Trypan blue and MTT assay whereas 14 days for the clonogenic assay. Notably, the MTT assay of HepG2 cells was not efficient at a cell density of 1 × 10^4 cells/well. In the MTT assay, HepG2 cells were usually seeded at a cell density of > 1 × 10^4 cells/well on 96 well-plates in cell biology experiments [22-25]. In this study, it was found that optimal cell density for the effective MTT assay in HepG2 Cells was a 1 × 10^3 cells/well on 96 well-plates in order to measure cell viability loss caused by exposure of radiation. This result indicated that it is necessary to consider the optimal cell density for radiation-induced cell viability test using the MTT assay.

In summary, radiation-induced cell viability of HepG2 cells was investigated as follow;

1) In both the MTT and Trypan blue assays, the cell viability was decreased in a dose-dependent manner and 5 Gy irradiation induced 50% of cell viability loss.

2) An incubation time (3 days) for both assays was shorter than that (14 days) of the Clonogenic assay.

3) For the effective MTT assay on 96 well-plates, an optimal cell density was 1 × 10^3 cells/well.

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Conflicts of interest: *none to declare.*

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