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EVALUATION OF MCF-7 CELL VIABILITY BY LDH, TRYPAN BLUE AND CRYSTAL VIOLET STAINING ASSAYS

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ABSTRACT

Viability of cultured mammalian cells is evaluated by a variety of techniques. In this study, experimental results of fast cell viability assays were compared to reveal the most suitable method for determination of hyperthermia effect on viability of human breast cancer Michigan Cancer Foundation-7 (MCF-7) cell line. The cells were exposed to heat at 42˚C for 2 hours to estimate the percentage of cell viability using four assays (trypan blue, lactate dehydrogenase (LDH), and crystal violet, (CV). There was a mild decrease in percentage of cell viability as the duration of heat exposure increased. Of the three counting techniques, the crystal violet nuclei showed consistent and significantly higher value (70.58±1.97) than trypan blue and LDH assay (81.07±20.12 and 77.06±11.84 respectively) ($p< 0.05$). This study reveals that CV was the most sensitive assay for adherent cell. It is also very effective; simple; and permits many samples to be analyzed rapidly and simultaneously.

Keywords: Cell viability, crystal violet, hyperthermia, lactate dehydrogenase, MCF-7, trypan blue

INTRODUCTION

A variety of methods have been used for evaluating the cytotoxic effects of cultured cells. One of these techniques, hemocytometer counting using the trypan blue dye is a widely used assay for staining dead cells (Philips, 1973).The total cell concentration and percentage of viability can be determined, as viable cells are distinguished from non-viable cells incorporate the blue stain (Berry *et al.,* 1996). However, trypan blue staining cannot be used to differentiate between healthy cells and cells that are alive but losing cell functions. This method also does not measure lysed cells. Moreover, adherent cells lend themselves less well to this method as they first have to be physically or enzymatically removed from the growth surface. Thus, the most common method used with adherent cells has been the crystal violet assay (Sanford *et al.,* 1951). Fixation of crystal violet staining can be done either using 4% paraformaldehyde or 1% glutaraldehyde. This staining procedure is very effective because it is simple; reproducible assay of cytotoxicity; and a number of materials can be evaluated simultaneously (Itagaki *et al.,* 1991; Saotome *et al.,* 1989). This assay is based on loss of ability to maintain and provide energy for metabolic growth and function which reflected by the colorimetric determination of the stained cells (Chiba *et al.,* 1997).

Another assay is used to assess cell viability is lactate dehydrogenase (LDH) assay. The release of LDH activity is related on total number of dead and lysed cells. Besides that, there is an assumption for LDH assay as follow: intracellular enzymes are rapidly released after damage to the cell membrane but this assumption is not necessarily correct. Furthermore, the stability of LDH can vary considerably, ranging from the loss of a few percent per day to a half-life of 12h depending upon the cell type. Therefore, crystal violet staining for measuring loss of monolayer adherence is the most sensitive assay, as compared to other non-clonogenic assays including trypan blue exclusion and LDH assay (Mickuvenie *et al.,* 2004). In the present study, we compared the results of the methods using trypan blue, crystal violet staining and LDH assay to determine the effect of hyperthermia on viability in human breast cancer MCF-7 cells.

MATERIALS AND METHODS

Cells

The MCF-7 human breast cancer cell line was obtained from American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium supplemented with 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 1% penicillin and 10% fetal calf serum (FCS). The cells were maintained in T25 flasks at 37°C and 5% CO2 humidified incubator. They were subcultured by 1% trypsin-EDTA (Gibco BRL, Life Technologies).

Hyperthermic exposure

MCF-7 cells, 1×104 cells/well in 200µl culture medium, were seeded in each well of 96-well plates. The cells were pre-cultured at 37˚C overnight incubation. To study the cytotoxicity of cells, hyperthermic exposure was carried out by placing culture plates in an incubator maintained at 42°C. Well temperature was monitored and maintained within 0.1°C during the treatment period. Cultured cells that were maintained at 37°C served as controls for all experiments. Cultured cells were subjected to 0.5, 1, 2, 3 or 4 hours of hyperthermic exposure for each temperature.

Observation under phase contrast microscope

After hyperthermia treatment, MCF-7 cells were observed using a Nikon phase contrast microscope. The photographs were taken at 10X /0.03 magnification.

Crystal violet assay

After hyperthermic exposure, the cultivation medium was removed gently from the wells and cells were washed with phosphate buffer saline (PBS). Non-adherent cells were washed off and remaining cells were fixed with 200µl of 4% paraformaldehyde for 30 minutes. After washing, 100 µl of 0.05% crystal violet (CV) solution in 20% ethanol was added and cells were allowed to stain for 30 minutes. Following three washes with distilled water, the plates were aspirated and allowed to air-dry at room temperature. To each well, 200µl of 10% acetic acid was added and incubated for 20 minutes with shaking. 100 µl of the dissolved dye solution was taken out and diluted in (1:4) distilled water. The optical density at 570nm at each well was measured on a microplate reader. 10% acetic acid was used as blank. The average absorbance of the control cells exposed to free culture medium was set to represent 100% of viability and the results were expressed as percentage of these controls (Ito, modified 1984).

Lactate dehydrogenase assay

LDH activity was measured in the incubation medium and the cells. The culture medium was removed culture plates placed on ice. Then, the plates were washed with cold phosphate buffered saline (PBS). One ml of a 1.35 % solution of Triton X-100 was added in 0.03 M sodium phosphate buffer, pH 7.4, and placed on ice. After 5 minutes, the solubilized cell lysate was removed and vortexed for 5 minutes. Aliquots of the medium (100 µl) or cell lysate (25 µl) were added to 0.03 M sodium phosphate buffer pH 7.4, containing 0.1 mM NADH and 0.25 mM pyruvate to the final volume of 1 ml and assayed by monitoring the rate of loss of NADH absorption at 515 nm on a microplate reader after 100 µl sample was transferred into each well. The enzyme activity of the medium and cell lysate was added together to find out the total activity. Cell viability was expressed as the percentage of the LDH activity in the culture medium, which was calculated by dividing the amount of activity in the medium by the total activity (Mickuvenie *et al.,* 2004).

Trypan blue

Trypan blue stain was prepared freshly as a 0.4% solution in 0.9% sodium chloride before each experiment was started. MCF-7 cells in 96 wells were washed in PBS and dispersed with 0.025% trypsin-EDTA. After trypsinization, 20 µl cell suspension was added to 20 µl of trypan blue solution and mixed thoroughly. Triplicate wells of viable cells were counted using a hemacytometer and the experiment was repeated three times. Cell viability was expressed as the percentage of cells staining blue (Berry *et al.,* 1996).

Statistical analysis

Results were expressed as a mean ± standard error of the mean (SEM) (SEM was within 5% of the average). The mean values were calculated from data taken from three different experiments performed in triplicates on separate days using freshly prepared reagents for all cases. When not shown, error bars lie within symbols. Significance testing was performed where indicated

using one-factor analysis of variance (ANOVA). The differences were evaluated significant at $p< 0.05$.

Result

Effect of hyperthermia on cell viability of MCF-7 was evaluated using 3 different assays (LDH, crystal violet assay with 1% glutaraldehyde fixation, crystal violet staining with fixation of 4% paraformaldehyde and trypan blue exclusion method).

Cells were treated at heat 42˚C for 1, 2 and 4 hours to determine the percentage of cell viability. The results were summarized in Figure 1. After 1 hour of hyperthermia exposure, the least loss of MCF-7 viability was measured by trypan blue (98.74±4.76 % viability) and the highest was determined by crystal violet assay with 1% glutaraldehyde and 4% paraformaldehyde fixation (92.13±6.00 and 93.33±3.21). After 2 and 4 hours of heat shock treatment at 42˚C, 89.99±4.45 and 69.85±4.44 respectively was the highest percentage of cell viability using crystal violet staining technique (1% glutaraldehyde). In contrast, 98.08±6.04 and 81.07±20.12 81.07±20.12 was lowest percentage value for estimation of cell viability by trypan blue assay. The crystal violet assay with 1% glutaraldehyde gave similar results as the crystal violet staining with 4% paraformaldehyde fixation. The most sensitive assays were those registering the cells still attached to the well surface meanwhile the least sensitive ones were tests of cell membrane integrity (Mickuvenie *et al.,* 2004). Of the 3 counting techniques, the crystal violet nuclei displayed consistent and significantly higher value (70.58±1.97) than trypan blue and LDH assay (81.07±20.12 and 77.06±11.84 respectively) ($p< 0.05$). The percentage of viability was significantly decreased after 1 and 2 hours of heat exposure at 42˚C compared to control (100% viability). There was a mild decrease in percentage of cell viability as the duration of heat exposure increased from 1 to 4 hours for each assay. More cells were killed at high temperature (42˚C) and longer duration (4 hours). However, temperatures above 42˚C and longer exposure of heat might cause damages in healthy cells (Mostafa *et al.,* 2007). (Figure 1)

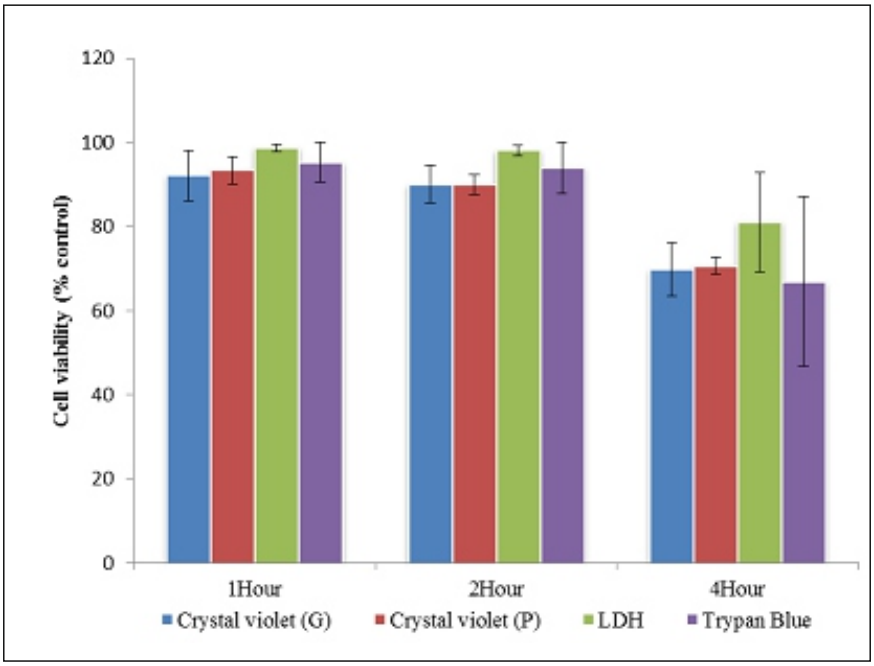

Figure 1: Effect of hyperthermia on viability of MCF-7 cell line. Cell death was measured by lactate dehyrogenase assay (LDH), crystal violet staining assay with fixation of 1% glutaraldehyde, crystal violet method with 4% paraformaldehyde fixation and trypan blue technique after hyperthermic exposure of 1 hour, 2 hours and 4 hours. Cells were treated in triplicate and the assays repeated on three separate experiments, shown as average ± SD (SD was within 5% of the mean). The technique using crystal violet, displayed significantly higher value than trypan blue and LDH assay at $p< 0.05$.

The cell viability was evaluated after hyperthermic exposure of 2 hours by three assays, in total. The value assays are measured continuously from day 1 to day 7. The results were shown in Figure 2. After 7 days of heat exposure at 42˚C, CV assays with 1% glutaraldehyde and 4% paraformaldehyde fixation showed less viability of the treated cells (75.22±3.47% and 74.11±1.11%, respectively) while trypan blue indicated higher viability (74.11±1.11%). The sensitivity of CV assay was significantly higher than LDH and trypan blue exclusion with 1% glutaraldehyde fixation method was not significantly different from those by CV with 4% paraformaldehyde. LDH and trypan blue methods have difference. Referring to Figure 2, LDH assay estimated lower cell viability than trypan blue assay after 2 hours of hyperthermic exposure at 42˚C; which indicated that LDH technique was more sensitive than trypan blue procedure. The percentage of cell viability was slightly decreased from day 1 to day 7 after the cells were treated with heat at 42˚C for 2 hours. (Figure 2)

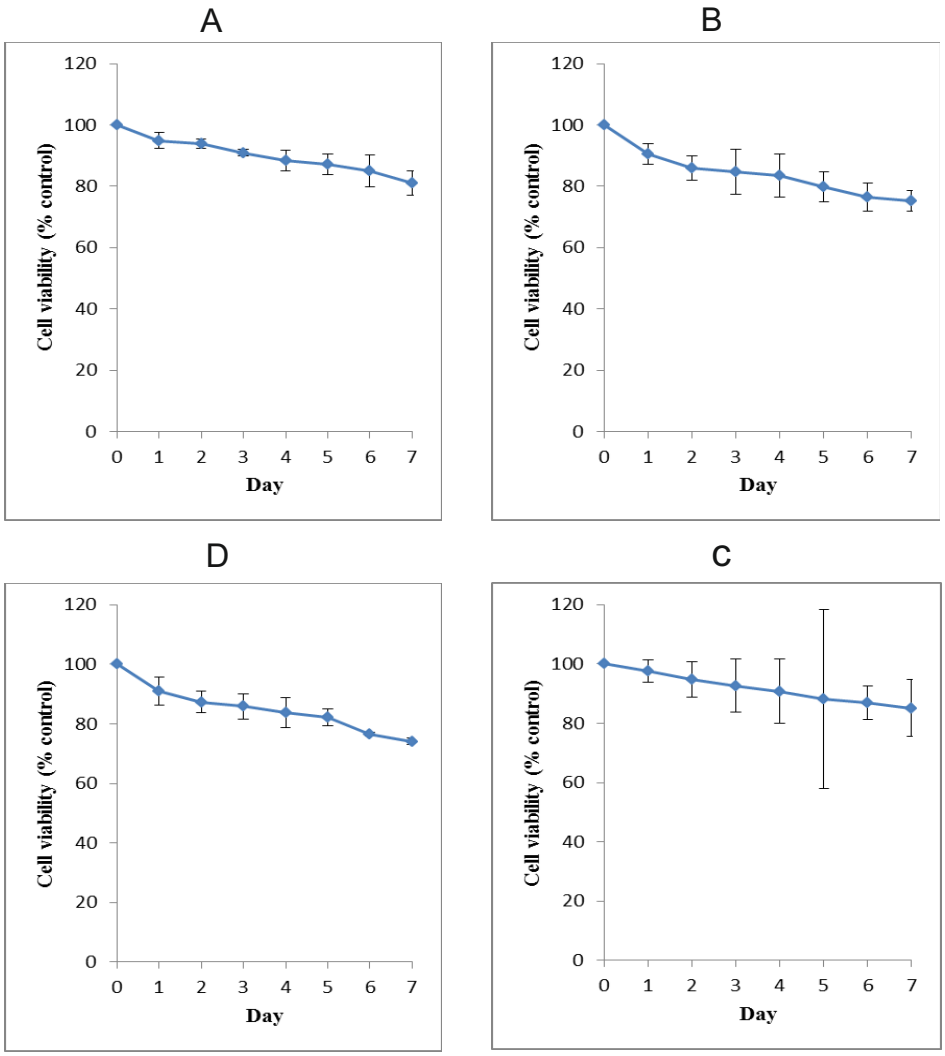

Figure 2: After 2 hours of hyperthermic exposure, percent cell viability of MCF-7 was determined by 3 different assays. The values assays were measured from day 1 to day 7. The data are presented as mean ± SD (SD was within 5% of the mean) from three independent experiments in triplicate. A) Lactate dehydrogenase assay (LDH). B) Crystal violet staining assay with 1% glutaraldehyde fixation. C) Crystal violet staining technique with 4% paraformaldehyde fixation. D) trypan blue exclusion method. Sensitivity of CV assay was significantly higher than LDH and trypan blue exclusion method at $p<0.05$.

DISCUSSION

It is assumed that a damaged membrane will be permeable to large charged molecules either from the outside or inside the cell to relate viability and plasma membrane. Trypan blue is the most popular vital exclusion dye for mammalian cells in monolayer culture as well as in suspension although limitations have been noted even in suspension (Krause *et al.,* 1984). The optimal density in cultures can be limited by cell death which is a significant parameter (Akatov *et al.,* 1985). Counting using trypan blue exclusion method underestimates cell death because they missed lysed cells (Sebastien *et al.,* 2001). Standard deviation of trypan blue technique was highest (80.96±30.30) compared to other assays which means that there might be error in counting number of cells. Cells that were attached to each other or clumps after trypsinization were counted as single particles by hemocytometer. This would account for the apparent lower values for viable cell count. There was difficulty to count cells that were contacted with the enclosing boundaries. Furthermore, cells had to be counted within 5 minutes because the number of blue-staining cells (death cells) increases with time after addition of the dye. Moreover, the trypan blue method involved exhaustive trypsinization of anchorage-dependent cells from T-flask growth surfaces. Complete cell release dependence on time-course and enzyme concentration of trypsinization (Berry *et al.,* 1996). Therefore, it proved that this assay was less inaccurate than the other assays from this study.

One of a more accurate way to assess cell death is to measure the lactate dehydrogenase released into the medium; which is reflective of cellular damage (Racher *et al.,* 1990; Wagner *et al.,* 1991; Legrand *et al.,* 1992; Falkenhain *et al.,* 1998). The activity of LDH measured as the reduction of pyruvate to lactate. There was difference in result between the LDH and Trypan blue methods. According to Table 2, LDH assay registered lower cell viability than Trypan blue assay after 2 hours of heat exposure at 42˚C. In this study, LDH assay was shown to be more sensitive in evaluating the earlier damages of cell membrane than the trypan blue procedure. However, the disadvantage of this assay is the phenol red in culture medium results in high background (Lappalainen *et al.,* 1994). Background absorbance from this factor can be corrected by including a culture medium background control. Besides that, the stability of LDH can vary considerably depending upon the cell type. There is an assumption that LDH release occurs rapidly after damage to the cell membrane but this is not necessarily correct.

The crystal violet assay involves simultaneous cell lysis and the released nuclei to stain purple in a hypertonic solution (citric acid) (Berry *et al.,* 1996). The stained nuclei are measured by a microplate reader at absorbance 595 nm. This method has been widely used for monitoring mammalian cell cultures because of the fast and quantitative release of stained nuclei. 1.35% Triton-X was used to lyse cells at a maximal level (Eisenberg *et al.,* 2010). Percentage viability of MCF-7 cell line determined by the crystal violet method; were significantly and consistently higher than measured by either the LDH or trypan blue methods ($p<0.05$). Thus, LDH and trypan blue were less sensitive assay when compared to crystal violet staining method. The assessment of an effect on cell viability might be based on the specific assay used. This is due to each cytotoxicity assay has a particular intracellular biochemical process such as metabolic dysfunction or cell death. Therefore, the chosen assay might be specific to one treatment and much less sensitive to another.

Trypan blue exclusion method and LDH procedure underestimated the loss of cell viability because this assays require that cell wall of target cells should be lysed (Shin *et al.,* 2000). Therefore, in the observations of cell viability of MCF-7 after hyperthermic exposure, the crystal violet staining method more useful than LDH and trypan blue assays. This staining technique is very effective for adherent cells; it is simple; and permit many samples to be analyzed rapidly (Itagaki *et al.,* 1991; Saotome *et al.,* 1989).

CONCLUSION

In this study, it had been analysed that crystal violet assay was more useful and suitable method than LDH and trypan blue assays to determine the effect of hyperthermia on viability of MCF-7 cell line. Crystal violet assay can be a suitable method to measure the cell viability for different types of normal and cancer cell lines.

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