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Evaluation of Hyperthermia Effect on Cell Viability using Crystal Violet Staining, LDH and Trypan Blue Assays

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ABSTRACT

There is a variety of techniques for evaluating the viability of cultured mammalian cells. 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 (MDA-MB 231) and normal liver (WRL-68) cell lines. The cells were exposed to heat at 42˚C for 4 different duration of heat exposure (1, 2, 3 and 4 hours); and percentage of cell viability was determined using three different assays (trypan blue (TB), lactate dehydrogenase (LDH) and crystal violet, CV (1% glutaraldehyde or 4% paraformaldehye fixation)). Cell viability by the CV with 1% glutaraldehyde fixation method was not significantly different from those by CV with 4% paraformaldehyde. Crystal violet assay showed less viability of the treated cells meanwhile trypan blue indicated highest viability for both cell lines. Of the three counting techniques, the crystal violet assay gave consistently and significantly higher value than LDH ($p<0.03$) and trypan blue assay ($p<0.05$). This proved that crystal violet assay was the most sensitive assay whilst the least sensitive assay was trypan blue exclusion method. Therefore, crystal violet assay was more effective; simple; permits many samples to be analyzed rapidly and simultaneously when compared to LDH and trypan blue assays for MDA-MB 231 and WRL-68 cell lines.

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INTRODUCTION

Worldwide, cancer is a leading cause of death. Hyperthermia is a new therapeutic approach for cancer therapy [1]. It is used to raise the temperature of a region of the body affected by cancer up to 41.5-43°C with minimal or no injury to normal tissues. This is due to normal cells have a defense system against heat meanwhile cancer cells do not have it [2]. The recent progress in hyperthermia has stimulated attempts to establish protocols for combination of hyperthermia with conventional modes of cancer treatment such as radiotherapy, chemotherapy and surgery [3]. The acute lethal potencies of anti-tumour compounds could be closely associated with their cytotoxic effects *in vitro* since such chemicals mainly exert their toxicity through their effects on basal cellular functions. Therefore, finding for the optimal and most suitable protocol of hyperthermia, cytotoxicity analysis *in vitro* could be the initial point [4,5]. A variety of methods have been used for evaluating the cytotoxic effects of cultured cells. 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. In the present study, we compared the results of the methods using crystal violet staining (1% glutaraldehyde or 4% paraformaldehye fixation), LDH and trypan blue assay to reveal the most suitable assay to determine the effect of hyperthermia on viability of human breast cancer (MDA-MB 231) and normal liver (WRL-68) cells.

MATERIALS AND METHODS

Cells:

The human normal (WRL-68) and breast cancer (MDA-MB 231) cell lines were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin and 10% fetal calf serum (FCS). Cells were maintained in a 5% CO_2 humidified incubator at 37°C.

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Hyperthermic exposure:

WRL-68 and MDA-MB 231 cells, 1×10^4 cells/well in 200µl culture medium, were seeded in each well of 96-well plates and pre-cultured overnight at 37˚C. Then, hyperthermic exposure was performed by placing culture plate in an incubator maintained at 42°C for studying cytotoxicity of cells. Well temperature was monitored and maintained within 0.1°C during the treatment period. Cultured cells were maintained at 37°C served as controls for all experiments. Cultured cells were subjected to 1, 2, 3 or 4 hours of hyperthermic exposure at 42°C.

Observation under phase contrast microscope:

After hyperthermia treatment, WRL-68 and MDA-MB 231 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 [6]. Percentage cell viability was calculated using the following equation: % cell viability=(Optical density of hyperthermia treated cells/Optical density of untreated cells)X100.

Lactate dehydrogenase assay:

After overnight incubation, LDH activity was measured in the medium and the cells. Then, the plates were washed with cold PBS. 1 ml of a 1.35 % solution of Triton X-100 was added in 0.03 M sodium phosphate buffer, pH 7.4, and the plates were placed on ice. After 5 minutes, the solubilized cell lysate was removed and vortexed. 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 [7]. The equation as follows: % cell viablity= (LDH activity in the medium/total LDH activity) X 100, where total activity is LDH in the supernatant plus LDH in the cell lysate.

Trypan blue assay:

Trypan blue stain was prepared freshly as a 0.4% solution in 0.9% sodium chloride before each experiment was started. 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 haemocytometer and the experiment was repeated three times. Cell viability was expressed as the percentage of cells staining blue [8].

Statistical analysis:

All data were expressed as a mean ± standard error of the mean (SEM). The mean values were calculated from data taken from four 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 $p < 0.05$.

RESULTS AND DISCUSSION

In the present study, hyperthermia-induced cytotoxicity was assessed using the three different assays which confirmed that hyperthermia stress greatly decreased cell viability of MDA-MB 231 cells with increasing duration of heat exposure (from 1 to 4 hours) meanwhile WRL-68 cells were maintained the same number as before hyperthermia treatment (as control) from 37° C to 42° C for 2 hours. However, there was a mild decrease in sum of WRL-68 viable cells as the duration of heat exposure increased from 2 to 3 hours at temperature of 42°C. In this study, MDA-MB 231 cells were started to die at temperature of 42°C for 1 hour whereas WRL-68 cell line was started to show cytotoxicity at 42°C for 3 hours of hyperthermic exposure (Fig. 1). It was

reasonable because MDA-MB 231 cell line sensitive to heat, therefore the facilitating effect of hyperthermia to cancer cell was obvious. This was due to MDA-MB 231 cells do not have defense system against heat, as do WRL-68 cells [1]. Therefore, 42°C for 2 hours was the most suitable temperature and duration of heat exposure to kill cancer cells (MDA-MB 231) without damaging normal cells. When the temperature and duration exposure continued to increase, the cellular proteins denature at that high temperature and the cell dies passively (necrosis) before initiation of apoptosis for MDA-MB 231 cell line. In this study, normal cell line might die exponentially by induction of apoptosis but they might not reach until necrosis.

After 1, 2, 3 and 4 hours of heat exposure, the least loss of MDA-MB 231 viability was measured by trypan blue meanwhile the highest was determined by crystal violet assay (Fig. 1). This was because 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 [8]. Furthermore, there might be error in counting number of cells because the standard error for trypan blue technique was highest when compared to other assays from this study (Fig. 1). Cells that were attached to each other or clumps after trypsinization were counted as single particles by haemocytometer. This would account for the apparent lower values for viable cell count. Therefore, it proved that this assay was less inaccurate than the other assays from this study.

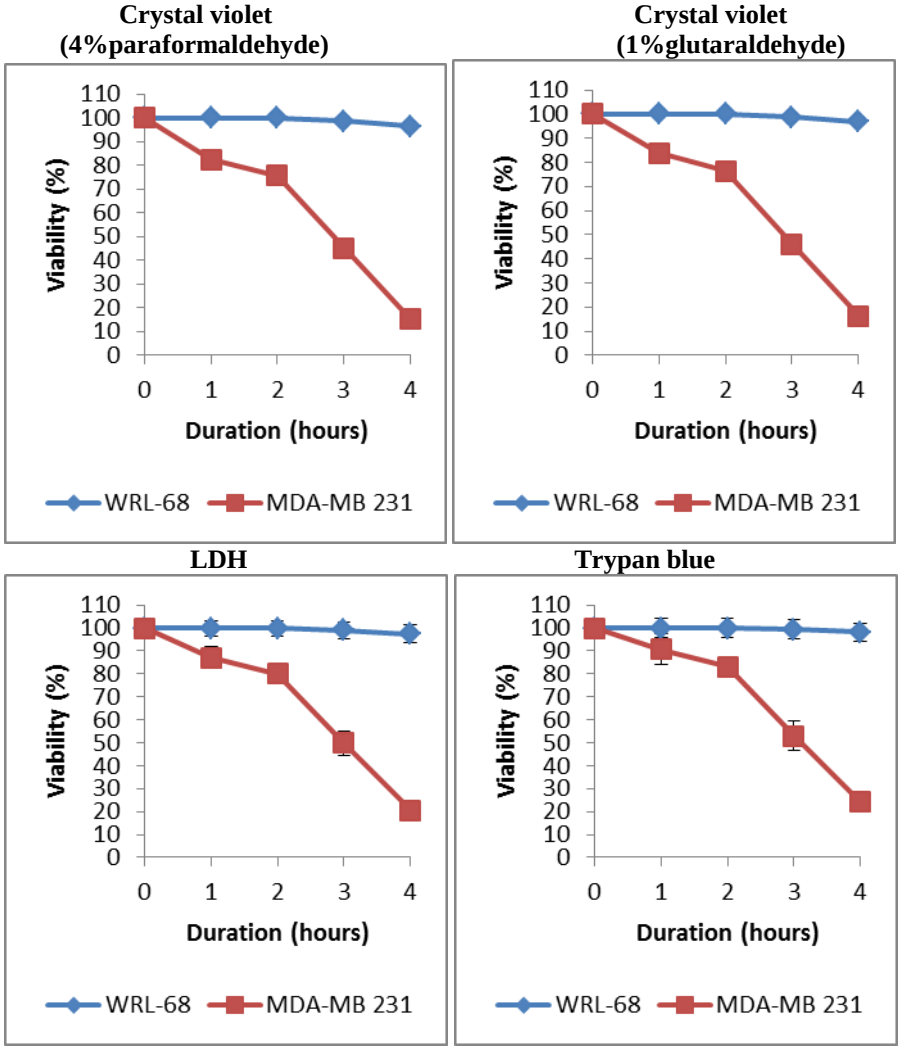

Fig. 1: After 2 hours of hyperthermic exposure at 42˚C, percentage cell viability of WRL-68 and MDA-MB 231 was determined by 3 different assays. The values assays were measured from 1 to 4 hours of duration heat exposure. The data are presented as mean ± SD (SD was within 5% of the mean) from three independent experiments in triplicate.

Cell viability by the CV with 1% glutaraldehyde fixation method was significantly same as CV with 4% paraformaldehyde ($p<0.001$). LDH assay estimated lower percentage cell viability than trypan blue assay. However, the disadvantage of this assay is the phenol red in culture medium results in high background [9]. Besides that, the stability of LDH can vary considerably depending upon the cell type.

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Table 1: Cell viability of WRL-68 and MDA-MB 231 following heat treatment[a]

Cells	Time(hours)	CV(4%paraformaldehyde)	CV(1%glutaralehyde)	LDH	TB
WRL-68	0	100±0 [b]	100±0 [b]	100±2.78 [b]	100±4.07 [b]
	1	100±0	100±0.19	99.88±3.25	100±4.07
	2	100±0	100±0	99.77±3.13	100±3.95
	3	98.77±0.10[e]	98.56±0.10[e]	95.06±3.83[d]	96.30±4.07[c]
	4	96.86±0.10[e]	96.55±0	92.45±3.83[d]	94.26±3.95[d]
MDA-MB 231	0	100±1.77 [b]	100±1.47 [b]	100±2.88[b]	100±3.73[b]
	1	82.32±2.53[e]	83.74±1.89[e]	87.02±4.61[c]	90.72±6.75[c]
	2	76±1.89[e]	76±1.89[e]	79.95±4.38	83.13±4.58[d]
	3	45.02±3.06	46.07±3.15	49.77±5.42[d]	53.01±6.39[c]
	4	15.35±1.89[e]	16.16±2.83[e]	20.51±3.92[d]	24.22±3.86

[a] Values represent mean±SD of three measurements from three independent experiments
[b] The viability of WRL-68 and MDA-MB 231 at 37°C as control
[c] $p<0.05$
[d] $p<0.01$
[e] $p<0.001$

Similar observations as MDA-MB 231 were made for WRL-68 cells too (Fig. 1); after 1, 2, 3 and 4 hours of hyperthermia exposure, crystal violet assay showed less viability of the treated cells whilst trypan blue indicated higher viability. However, the sensitivity of three assays for WRL-68 cell line was significantly same as the sensitivity for MDA-MB 231 cell line (CV: $p<0.001$; LDH; $p<0.03$; TB: $p<0.05$). 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 [6].

Of the three counting techniques, the crystal violet assay gave consistently and significantly higher value than LDH ($p<0.03$) and trypan blue assays ($p<0.05$) for both cell lines (MDA-MB 231 and WRL-68) (Fig. 1).[10], crystal violet method is more sensitive and earlier predictor of toxicity than classical LDH or neutral red measurements. In addition, it is very effective for adherent cells. Therefore, it was the most sensitive assay whereas the least sensitive assay was trypan blue exclusion method for MDA-MB 231 and WRL-68 cells lines.

Conclusion:

In this study, it was clearly proved 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 MDA-MB 231 and WRL-68 cell lines. It is simple; rapid and reproducible method than other assays used in this study.

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