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HEAT SENSITIVITY BETWEEN HUMAN NORMAL LIVER (WRL-68) AND BREAST CANCER (MCF-7) CELL LINES

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Abstract- Hyperthermia is an innovative method for various types of cancer treatments which has potential to increase cytotoxic effect within the tumor volume, without increase normal tissue toxicity. In the present study, it was aimed to investigate temperature and duration of heat shock on viability of human normal liver (WRL-68) and breast carcinoma (MCF-7) cell lines using MTT and methylene blue assay. These cells were exposed to heat at three different temperatures (38, 40 and 42°C) for five different duration of heat exposure (0.5, 1, 2, 3 and 4 hrs.). Viability of WRL-68 and MCF-7 cell lines is highly influenced by temperature and duration of hyperthermic exposure. After increasing temperature from 38 to 42°C, more cancer cells were killed during the same period of heat exposure compare to normal cells. This was due to MCF-7 cells do not have defense system against heat, as do WRL-68 cells. There was a great variation of thermo sensitivity among normal cell and cancer cell. In this study, it was clearly observed that WRL-68 cells were killed at 42°C for 3 hrs. meanwhile MCF-7 cells need temperature of 38°C for 0.5 hrs. In future, more synergistic effects of combining hyperthermia with chemotherapy, radiotherapy, immunotherapy or gene therapy must be study in order to develop better therapeutic effects in breast cancer treatment.

Keywords- Hyperthermia, WRL-68, MCF-7, MTT assay, Methylene blue assay, temperature, duration exposure, thermo sensitivity

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Introduction

Hyperthermia is an innovative method for various types of cancer treatments amongst chemotherapy, radiotherapy, surgery, gene and immunotherapy [1]; which has potential to increase cytotoxic effect within the tumor volume, without increase normal tissue toxicity. This is due to normal cells have a defense system against heat meanwhile cancer cells do not have it [2]. Hyperthemia kills cancer cells by heating them to few degrees above physiological temperatures (>37°C) [3,4].

In the hyperthermic region above the maximum growth temperature causes significant cellular responses such as changes in stability of cell membrane, shape, membrane potential, pH, metabolism, nuclear and cytoskeleton structures, macromolecular synthesis, intracellular signal transduction in hormone-receptor interactions, induction of heat shock proteins, gene expression, chromatin organization and synthesis of DNA, RNA, protein and enzymes as well as in cell adhesion [5]. Moreover, it is usually believed that the hyperthermia toxicity is most directly correlated to nucleolic, cytoplasmatic or membrane protein denaturation and cell damage is the most direct effect of hyperthermia toxicity [6-9].

Treatment outcome varied hugely between different types of cell lines although same settings of hyperthermia used. On the other hand, small changes in hyperthermia settings could influence greatly difference in the final treatment outcome. Therefore, viability of normal and cancer cells is highly influenced by the temperature and duration exposure of heat (hyperthermia settings).

Induction of apoptosis for human fibrous histiocytoma cells was inhibited by hyperthermia treatment at 43°C for 1 hour, a temperature lower than that was not able to induce apoptosis although increase the duration of heat exposure. However, Yonezawa and co-workers observed that necrotic death was occurred at temperature of 46°C for 1 hour where cell death is passive; and involves lysis formation from the damaged cell and the release of its cellular content to the surrounding environment [10]. In contrast, heat shock at 44°C inhibited induction of apoptosis significantly in normal fibroblasts cells [11]. Therefore, a different mechanism was involved in heat shock induced cell death among normal cell and cancer cells [3]. According to Omar and Lanks (1984) [12] investigated that cancer cells are more susceptible to killing by heat than normal cells after the hyperthermia treatment (43-45°C). According to Watanabe and Suzuki (1989) [13], normal cells have reduced heat sensitivities 5-6 times at growing condition meanwhile the cancer did not. Heat shock proteins are synthesized by a set of

inducible heat shock genes which is activated by hyperthermia [14]. They act as molecular chaperons in connection by hyperthermia or as markers of thermal injury [15]. Moreover, they protect cells from thermal damage and enhance cellular recovery from lethal heat shock [16,17].

In the present study, it was aimed to investigate temperature and duration of heat shock on viability of human normal liver (WRL-68) and breast carcinoma (MCF-7) cell lines determined with MTT and methylene blue assay using three different temperatures (38,40 and 42°C) for five different duration of heat exposure (0.5,1,2,3 and 4 hrs.).

Methodology

Cells

The human breast cancer cell line MCF-7 and normal liver cell line WRL-68 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, 100 mg/mL streptomycin, and 10% fetal calf serum (FCS). Cells were maintained in a 5% CO_2 humidified incubator at 37°C.

Hyperthemic Exposure

MCF-7 and WRL-68 cells, 1×10^4 cells/well in 200µl culture medium, were seeded in each well of 96-well plates and were precultured overnight incubation at 37°C. Then, hyperthermic exposure was performed by placing culture plates in an incubator maintained at 38°C, 40°C and 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 0.5, 1, 2, 3 or 4 hrs. of hyperthermic exposure for each temperature.

Observation Under Phase Contrast Microscope

After hyperthermia treatment, WRL-68 and MCF-7 cells were analyzed under a Nikon phase contrast microscope at 10X /0.03 magnification to determine viable cells. Total number of viable cells was observed under phase contrast microscope.

MTT Assay

MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple colour. The assay was performed essentially as described by Alley et al. (1988) [18] with modification. The culture medium was removed from the culture plates after hyperthemic exposure and added with 20 µl of MTT media. The well plates were incubated for 4 hrs. at 37°C. The MTT media was aspirated slowly from the wells. 200 µl of dimethyl sulfoxide (DMSO) was added to each well. In addition, 25 µl of glycine buffer was added. Well plates were kept in dark for 15 minutes. Finally, absorbance at 570nm at each well was measured on a microplate reader. This value was then background subtracted (from media only wells) at 650nm and compared with controls, which are the values of cells without any treatment for obtaining cell growth. 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.

Methylene Blue Assay

Cell viability was measured by the methylene blue technique. The cellular protein assay was based on the method described by Finlay et al. (1984) [19] with modification. After heat shock treatment, the cultivation medium was removed gently from the wells and cells were washed with PBS. Non-adherent cells were washed off and remaining cells were fixed with 200µl of 4% paraformaldehyde for 30 minutes. After washing, 200 µl of 0.05% methylene blue (MB) 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. 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.

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 two 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

As shown in [Fig-1], [Fig-2], [Fig-3], [Fig-4], [Fig-5] and [Fig-6], MCF-7 and WRL-68 cells were treated by 38, 40 and 42°C incubator hyperthermia for 0.5,1,2,3 and 4 hrs. for each temperature.

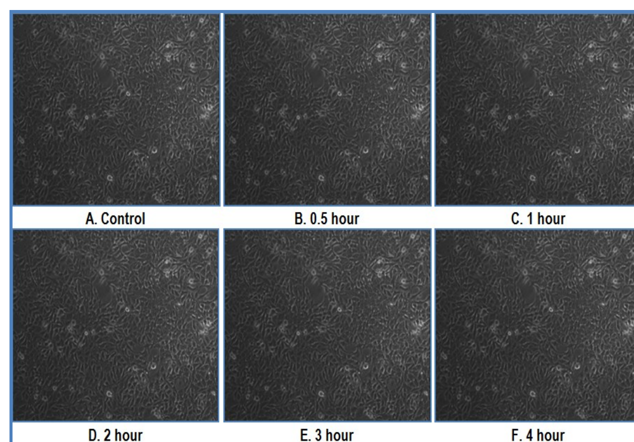

Fig. 1- WRL-68 cells after hyperthermia treatment. (A) WRL-68 cells without any treatment; (B-F) WRL-68 cells were treated by 0.5,1,2,3 and 4 hrs. at 38ºC incubator hyperthermia respectively.

Images were taken immediately after the hyperthermia treatment. Total number of viable MCF-7 cells decreased as increasing temperature and duration of heat exposure meanwhile WRL-68 cells were remained the same amount as before hyperthermia treatment (100% viability) from 37°C to 40°C for 0.5 to 4 hrs. There was a

mild decrease in total number of viable cells as the duration of heat exposure increased from 2 hrs. to 3 hrs. at temperature of 42°C. For MCF-7 cell, sum of viable cells was decreased significantly after incubator hyperthermia treatment. It was reasonable because MCF-7 cell line sensitive to heat, therefore the facilitating effect of hyperthermia to cancer cell was obvious. This was because MCF-7 cells do not have defense system against heat, as do WRL-68 cells [2]. WRL-68 might express heat shock-induced proteins to reactivate denatured proteins; degrade abnormally structured proteins; inhibit secretion of abnormal proteins and assisting the transfer of secretory proteins by blockage of folding [20]. Therefore, WRL-68 cells were survived in undesirable circumstances such as in this study (42°C for 2 hrs.) (refer to [Fig-5]).

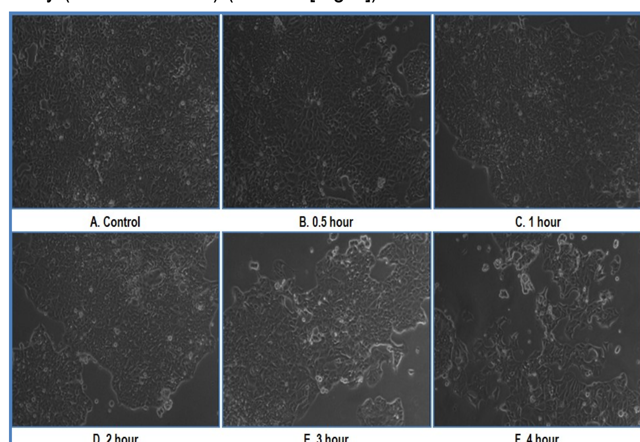

Fig. 2- MCF-7 cells after hyperthermia treatment. (A) MCF-7 cells without any treatment; (B-F) MCF-7 cells were treated by 0.5,1,2,3 and 4 hrs. at 38ºC incubator hyperthermia respectively.

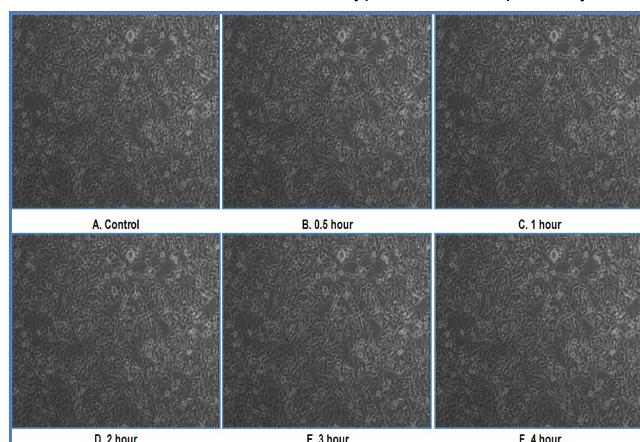

Fig. 3- WRL-68 cells after hyperthermia treatment. (A) WRL-68 cells without any treatment; (B-F) WRL-68 cells were treated by 0.5,1,2,3 and 4 hrs. at 40ºC incubator hyperthermia respectively.

However, cell death of WRL-68 cells were occurred when temperature increased to 42°C (>2 hrs. of heat exposure). WRL-68 and MCF-7 cells might die exponentially by induction of apoptosis where cell death is active.

When temperature and duration of heat exposure continued to increase, the distinct threshold for triggering necrosis is reached, heat shock protein expression might inhibited; the cellular proteins are denatured at that high temperature and the cells died traumatically or passively. Based on Gabai and Kabakov study (1993) [21], cytoskeleton damages and impairment of DNA repair system caused by hyperthermia treatment most likely occur during mitosis; therefore MCF-7 (cancer) cell is more susceptible to heat than WRL-68 (normal) cell as cancer cells undergo faster cell division than normal cells.

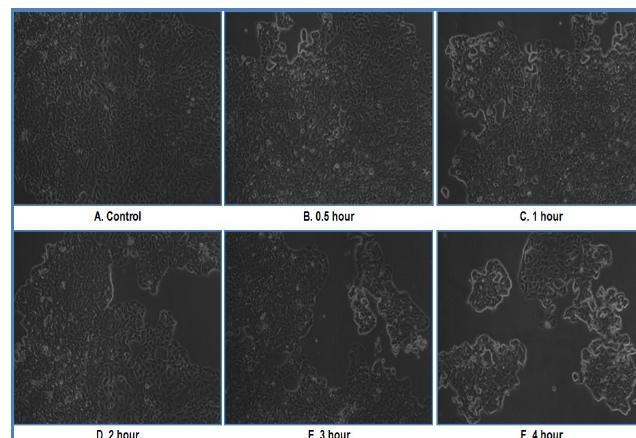

Fig. 4- MCF-7 cells after hyperthermia treatment. (A) MCF-7 cells without any treatment; (B-F) MCF-7 cells were treated by 0.5,1,2,3 and 4 hrs. at 40ºC incubator hyperthermia respectively.

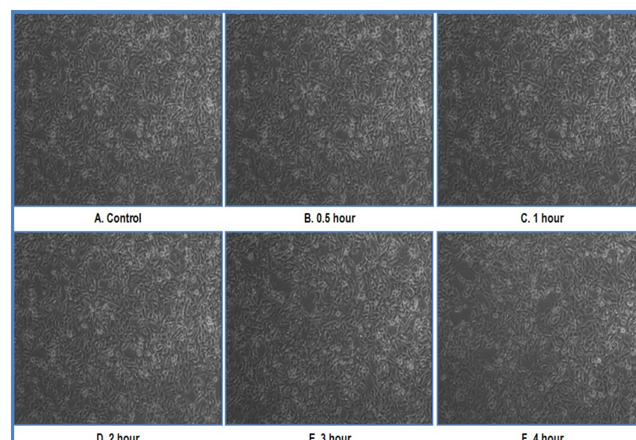

Fig. 5- WRL-68 cells after hyperthermia treatment. (A) WRL-68 cells without any treatment; (B-F) WRL-68 cells were treated by 0.5,1,2,3 and 4 hrs. at 42ºC incubator hyperthermia respectively.

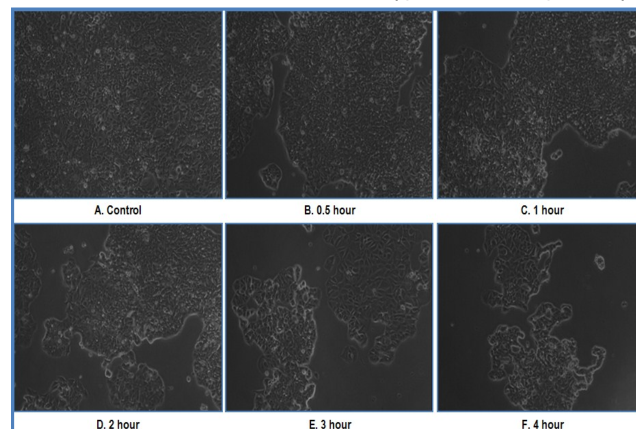

Fig. 6- MCF-7 cells after hyperthermia treatment. (A) MCF-7 cells without any treatment; (B-F) MCF-7 cells were treated by 0.5,1,2,3 and 4 hrs. at 42ºC incubator hyperthermia respectively.

Cell viability of WRL-68 and MCF-7 cells was determined using methylene blue and MTT assay. Cultured cells were maintained at 37°C served as control. Cell death causes change of colour from colourless to blue in well for methylene blue assay. The intensity of colour in wells increases as the total number of death cells increases. However, WRL-68 cells remained colourless until reached at 42°C for 3 hrs. of heat exposure where the cells started to die. MCF-7 cell line showed cytotoxicity at temperature of 38°C for 30 minutes of hyperthermic exposure. However, after increasing temperature from 38 to 42°C, more cancer cells were killed during the same period of heat exposure when compare to normal cells in this study. This was clearly observed through the colour intensity which was darker for MCF-7 cells than WRL-68 cells. This revealed that colour intensity increases as the temperature and duration of heat exposure increases. MTT assay also gave similar results as methylene blue assay except the colour changes because MTT assay was used to measure the cellular enzyme activity of mitochondriol succinate dehydrogenase that reduces the tetrazolium dye MTT (yellow colour) to its insoluble formazon (purple colour) [18]. In contrast, basophilic compounds such as proteins and nucleic acids which were quantified by methylene blue assay causes the colour changes from colourless to blue [20].

Based on [Fig-8], percentage viability of MCF-7 cells decreased sharply from 100% to (71.642±3.674) (p=0.041); (44.145±1.171) (p=0.05) and (17.865±0.436) (p=0.002) for 4 hrs. at temperatures of 38, 40 and 42°C respectively using MTT assay. On the other hand, WRL-68 cell viability which was measured by MTT assay; did not cause any decrease compared to control (100%) from 37°C to 40°C for 0.5 to 4 hrs. and 42°C for 0.5 to 2 hrs. (refer to [Fig-7]).

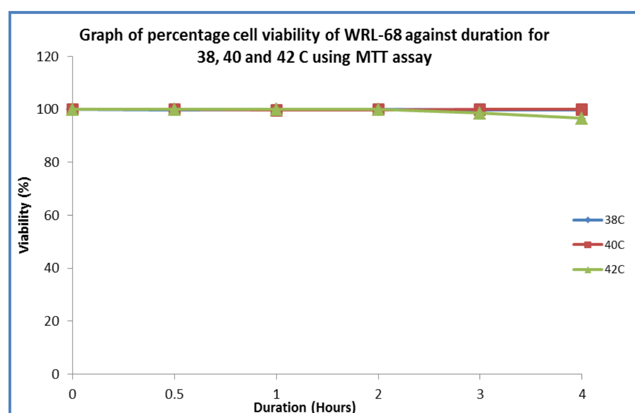

Fig. 7- WRL-68 cells after hyperthermic exposure for 3 different temperatures (38, 40 and 42°C), cell viability percentage was determined by MTT assay. The data are presented as mean ± SD (SD was within 5% of the mean) from one independent experiment in triplicate.

After that, there was a mild decrease in percentage viability from 42°C for 3 to 4 hrs. (98.560±0.001) (p=0.04) to (96.545±0.001) (p=0.048). The methylene blue technique gave similar results as the MTT assay.

According to [Fig-9], the percentage viability of WRL-68 cell remained same percentage as control (100% viability) until temperature of 42°C for 2 hrs. of hyperthermic exposure and then significantly decreased for methylene blue assay which were (98.762±0.001) (p=0.001) and (96.857±0.000) (p=0.05) at 42°C for 3 and 4 hrs. respectively. On the other hand, MCF-7 cell percentage viability was decreased from 100% viability to (71.889±3.111) (p=0.045), (44.686±2.626) (p=0.05) (19.317±3.887) (p=0.034) for 4 hrs. at temperatures of 38, 40 and 42°C respectively for methylene blue assay (refer to [Fig-10]).

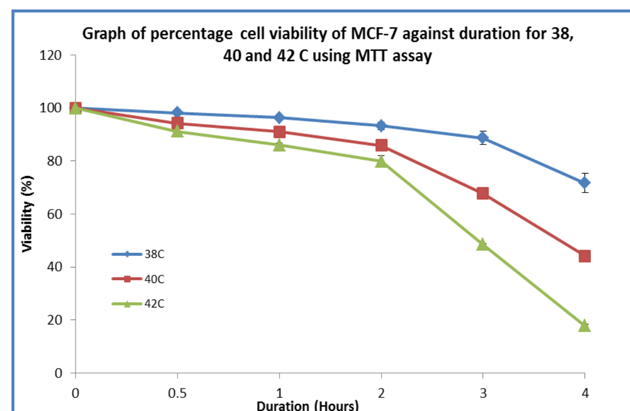

Fig. 8- MCF-7 cells after hyperthermic exposure for 3 different temperatures (38, 40 and 42°C), cell viability percentage was determined by MTT assay. The data are presented as mean ± SD (SD was within 5% of the mean) from one independent experiment in triplicate.

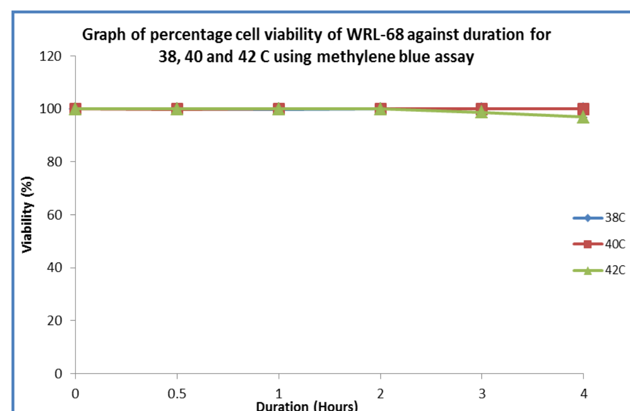

Fig. 9- WRL-68 cells after hyperthermic exposure for 3 different temperatures (38, 40 and 42°C), cell viability percentage was determined using methylene blue assay. The data are presented as mean ± SD (SD was within 5% of the mean) from one independent experiment in triplicate.

When exponentially growing cultured cells (WRL-68 and MCF-7) were exposed to a defined temperature between 38°C and 42°C of heat exposure, a temperature–effect curve was defined by plotting the percentage of cell viability against the duration of hyperthermia. In this study, treatment outcome varied greatly among human normal (WRL-68) and breast cancer (MCF-7) cell lines although same or different settings of hyperthermia used. Viability of normal and cancer cells is highly influenced by the temperature of the hyperthermia treatment and the duration of heating at that temperature (hyperthermia settings). For example, proliferation of human osteosarcoma cells was inhibited by hyperthermia treatment at 42°C. In this study, MCF-7 cells were started to die at temperature of 38°C for 0.5 hrs. meanwhile WRL-68 cell line was started to show cyto-

toxicity at 42°C for 3 hrs. of hyperthermic exposure. This proved that MCF-7 cells were killed by temperature of 42°C which was well-tolerated by normal cell line (WRL-68). Normal cells have reduced their heat sensitivity 5 to 6 times at growing condition but cancer cell did not [13]. HSP70 induction by hyperthermia treatment in cancer cells is lower than those in normal cells [13]. This causes the heat shock proteins unable to protect cancer cells from thermal damage and enhance cellular recovery from heat exposure. Therefore, MCF-7 cells died exponentially with induction of apoptosis whereas WRL-68 cells were survived in undesirable condition such as at 42°C incubator hyperthermia for 2 hrs. (refer to [Fig-7] and [Fig-9]).

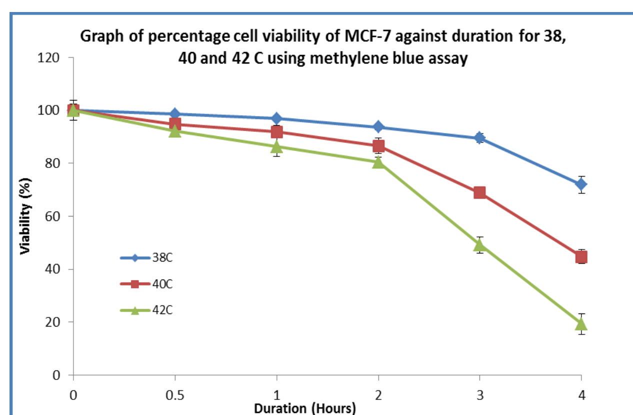

Fig. 10- MCF-7 cells after hyperthermic exposure for 3 different temperatures (38, 40 and 42°C), cell viability percentage was determined using methylene blue assay. The data are presented as mean ± SD (SD was within 5% of the mean) from one independent experiment in triplicate.

Although, cancer therapies such as chemotherapy and radiotherapy can induce tumour cell apoptosis but physiological stress conditions like growth factor, starvation, hypoxia or heat can also be equally effective to DNA damaging treatment [22]. Abnormalities of these environmental factors affect tumour cell proliferation and response to anti-tumour therapy. Tumour cells with a disorganized and compact vascular structure have difficulty dissipating heat. Thus, hyperthermia might cause cancer cells to undergo apoptosis in direct response to heat. In contrast, healthy cells can more easily maintain at a normal temperature. Based on Urano *et al,* 1983 [23] study, tumour cells might be more sensitive to lower temperature than normal tissue. Results from this study proved that Urano's hypothesis was right where MCF-7 cells were sensitive to temperature of 38°C (refer to [Fig-8] and [Fig-10]). Besides that, as shown in Song *et al* [24] study that rat muscle and skin have a greatly enhanced blood flow at temperatures above 42°C meanwhile tumours have a greatly reduced blood flow at these temperatures. Reduction of blood flow and blood vessel density, resulting in regions with hypoxia and low pH levels, which is not found in normal tissues under undisturbed conditions. Hypoxia might exhibit anaerobic metabolism with a resultant accumulation of lactic acid [25]. Acute acidification pH increases the rate of cell death by decrease heat shock protein levels [26].

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