Heat Sensitivity between Human Normal Liver (WRL-68) and Breast Cancer (MDA-MB 231) Cell lines

Asita. E, and Salehhuddin. H.

***Abstract*—**Hyperthermia is a potential tool for various types of cancer treatments in which body tissue is exposed to high temperature with usually minimal injury or no injury to normal tissues. 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 (MDA-MB 231) 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 hours). Viability of WRL-68 and MDA-MB 231 cell lines is dependent on 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 MDA-MB 231 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. It was observed that WRL-68 cells were killed at 42°C for 3 hours meanwhile MDA-MB 231 cells need temperature of 38°C for 0.5 hours.

Keywords*—Cell viability; hyperthermia; methylene blue assay; MTT assay*

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I. INTRODUCTION

Hyperthermia is a potential tool for various types of cancer treatments amongst chemotherapy, radiotherapy, surgery, gene and immunotherapy; in which body tissue is exposed to high temperatures (up to 45℃). The high temperatures can kill cancer cells or prevent their further growth with usually minimal injury or no injury to normal tissues [1]. This is because normal cells have a defense system against heat meanwhile cancer cells do not have it [2].

The mechanism of heat-induced cell death generates changes in fluidity of cell membrane, shape, 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 and RNA [3].

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 [4].

Treatment outcome varied greatly between different types of cancer cell lines although same settings of hyperthermia used. In addition, viability of normal and cancer cells is highly influenced by the temperature and duration exposure of heat. Proliferation of human osteosarcoma cells were inhibited by hyperthermia treatment at 42°C meanwhile heat shock at 44°C inhibited proliferation significantly in normal fibroblasts cells [5]. Therefore, a different mechanism was involved in heat shock induced cell death among normal cell and cancer cells [1]. According to Omar and Lanks (1984) [6] 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) [7], 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 [8]. They act as molecular chaperons in connection by hyperthermia or as markers of thermal injury [9]. Moreover, they protect cells from thermal damage and enhance cellular recovery from heat exposure [10, 11].

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 (MDA-MB 231) cell lines determined with MTT assay 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 hours).

II. METHODOLOGY

2.1 Cells

The human breast cancer cell line MDA-MB 231 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.

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2.2 Hyperthemic exposure

MDA-MB 231 and WRL-68 cells, 1×10^4 cells/well in 200µl culture medium, were seeded in each well of 96-well plates and were pre-cultured 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 hours of hyperthermic exposure for each temperature.

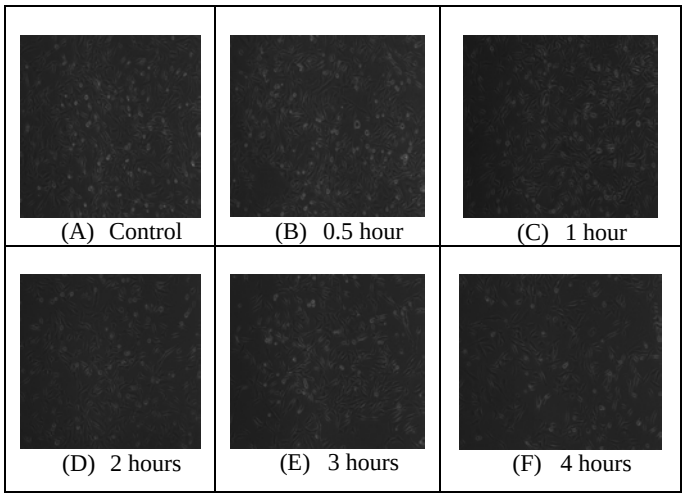
2.3 Observation under fluorescence microscope

After hyperthermia treatment, WRL-68 and MDA-MB231 cells were analyzed under a Nikon fluorescence microscope at 10X /0.03 magnification to determine viable cells. Total number of viable cells was observed under fluorescence microscope.

2.4 MTT Assay

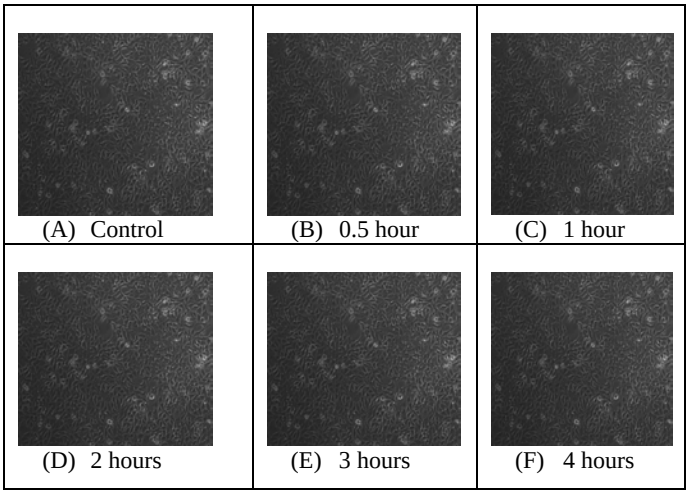
MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple colour. The assay was performed essentially as described by Alley *et al.* (1988) [12] with modification. The culture medium was removed from the culture plates after hyperthemic exposure and added with 20 µl of MTT media. The well plates were incubated for 4 hours 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.

2.5Methylene blue assay



Cell viability was measured by the methylene blue method. The cellular protein assay was based on the method described by Finlay et al. (1984) [13] with modification. After hyperthermic exposure, 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 min. After washing, 200 µl of 0.05% crystal violet (CV) solution in 20% ethanol was added and cells were allowed to stain for 30 min. 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 min 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.

2.6 Statistical analysis



Results were expressed as a mean ± standard error of the mean (SEM). 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 $p < 0.05$.

III. RESULTS & DISCUSSION

As shown in Figures 1, 2, 3, 4, 5 and 6, MDA-MB 231 and WRL-68 cells were treated by 38, 40 and 42°C incubator hyperthermia for 0.5,1,2,3 and 4 hours for each temperature. Images were taken immediately after the hyperthermia treatment.

Figure 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 hours at 38ºC incubator hyperthermia respectively.

Figure 2: MDA-MB 231 cells after hyperthermia treatment. (A) MDA-MB 231 cells without any treatment; (B-F) MDA-MB 231 cells were treated by 0.5,1,2,3 and 4 hours at 38ºC incubator hyperthermia respectively.

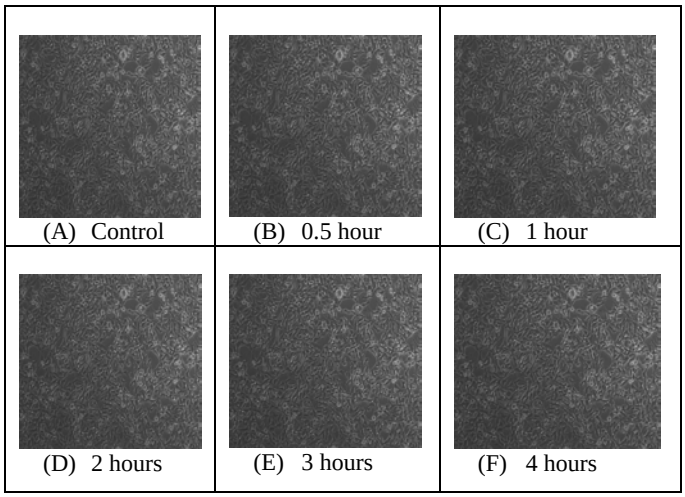


Figure 3: WRL-68 cells after hyperthermia treatment. (A) WRL-68 cells without any treatment; (B-F) WRL-68 cells were treated by 0.5,1,2,3 and 4 hours at 40ºC incubator hyperthermia respectively.

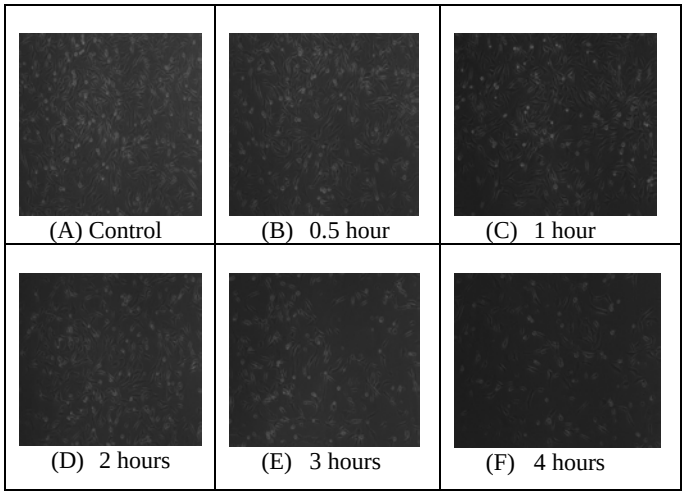


Figure 4: MDA-MB 231 cells after hyperthermia treatment. (A) MDA-MB 231 cells without any treatment; (B-F) MDA-MB 231 cells were treated by 0.5,1,2,3 and 4 hours at 40ºC incubator hyperthermia respectively.

Total number of viable MDA-MB 231 cells decreased as increasing temperature and duration of heat shock 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 hours. There was a mild decrease in total number of viable cells as the duration of heat exposure increased from 2 hours to 3 hours at 42°C. For MDA-MB 231 cell, sum of viable cells was decreased significantly after incubator hyperthermia treatment, which 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 [2].

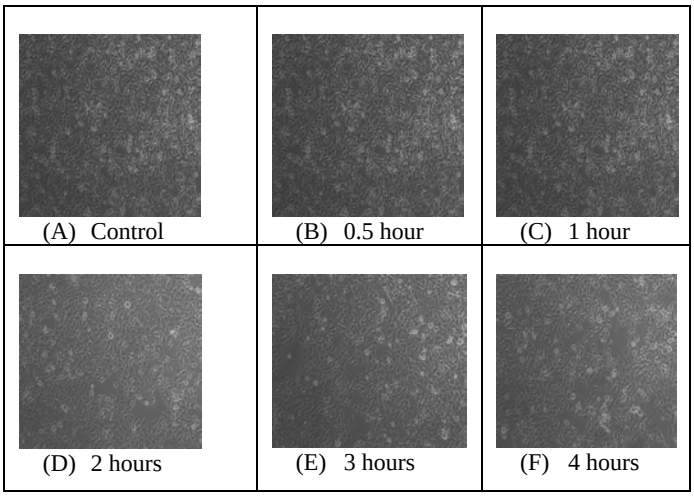


Figure 5: WRL-68 cells after hyperthermia treatment. (A) WRL-68 cells without any treatment; (B-F) WRL-68 cells were treated by 0.5,1,2,3 and 4 hours at 42ºC incubator hyperthermia respectively.

WRL-68 might be 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 [14]. Therefore, WRL-68 cells were survived in undesirable circumstances such as in this study (42°C for 2 hours) (refer to Figure 5). However, cell death for WRL-68 occurred when temperature increased to 42°C (>2 hours of heat exposure). WRL-68 and MDA-MB 231 cells might die exponentially by induction of apoptosis. When temperature and duration of heat exposure continued to increase, the distinct threshold for triggering necrosis is reached, heat shock protein expression might be inhibited; the cellular proteins are denatured at that high temperature and the cells died passively.

Cell viability of WRL-68 and MDA-MB 231 cells were 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 number of death cells increases. However, WRL-68 cells remain colourless until reached at 42°C for 3 hours of heat exposure where the cells start to die. MDA-MB 231 cell line starts to show 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 compare to normal cells in this study. This was clearly observed through the colour intensity which was darker for MDA-MB 231 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) [11]. In contrast, basophilic compounds such

as proteins and nucleic acids were quantified by methylene blue assay where the colour changes from colourless to blue [13].

According to Figure 10 and Figure 12, percentage viability of MDA-MB 231 cells decreased to (69.256±0.021) (p=0.000); (43.860±0.037) (p=0.000) and (16.159±0.027) (p=0.001) for 4 hours 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; maintained the same percentage as control (100%) from 37°C to 40°C for 0.5 to 4 hours and 42°C for 0.5 to 2 hours (refer to Figure 10). After that, there was a mild decrease in percentage viability from 42°C for 3 to 4 hours (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. As shown in Figure 9 and Figure 11, the percentage viability of WRL-68 cell remained same percentage as control (100% viability) until temperature of 42°C for 2 hours 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=0.052) at 42°C for 3 and 4 hours respectively. On the other hand, MDA-MB 231 cell percentage viability was decreased from 100% viability to (69.518±0.003) (p=0.045), (43.590±0.007) (p=0.05) (15.346±0.005) (p=0.064) for 4 hours at temperatures of 38, 40 and 42°C respectively.

When exponentially growing cultured cells (WRL-68 and MDA-MB 231) were exposed to a defined temperature between 38°C and 42°C of heat exposure, a dose–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 (MDA-MB 231) cell lines although same 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. In this study, WRL-68 cell line starts to show cytotoxicity at 42°C for 3 hours of hyperthermic exposure meanwhile MDA-MB 231 cells start to die at temperature of 38°C for 0.5 hours. This proved that MDA-MB 231 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 [10]. HSP70 induction by hyperthermia treatment in cancer cells is lower than those in normal cells [10]. This causes the heat shock proteins unable to protect cancer cells from thermal damage and enhance cellular recovery from heat exposure. Therefore, MDA-MB 231 cells died exponentially with induction of apoptosis meanwhile WRL-68 cells were survived at certain temperature and duration of heat exposure (refer to Figure 9 and 11).

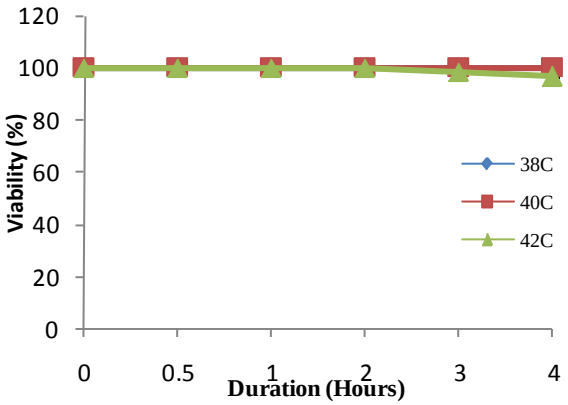


Figure 9: WRL-68 cells after hyperthermic exposure for 4 different temperatures (38, 40 and 42°C), cell viability percentage was determined by MTT assay. The data are presented as mean ± SD (SD was within 5% of the mean) from one independent experiment in triplicate.

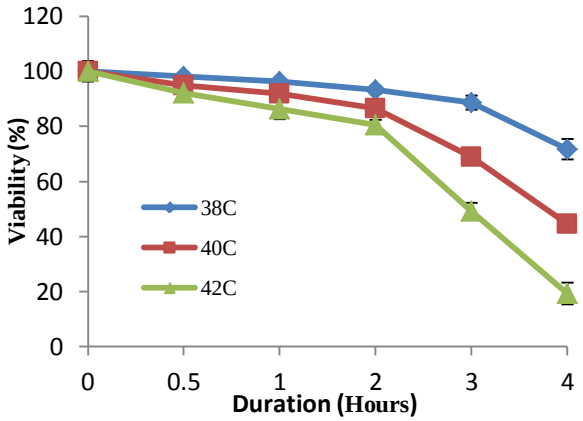


Figure 10: MDA-MB 231 cells after hyperthermic exposure for 4 different temperatures (38, 40 and 44°C), cell viability percentage was determined by MTT assay. The data are presented as mean ± SD (SD was within 5% of the mean) from one independent experiment in triplicate.

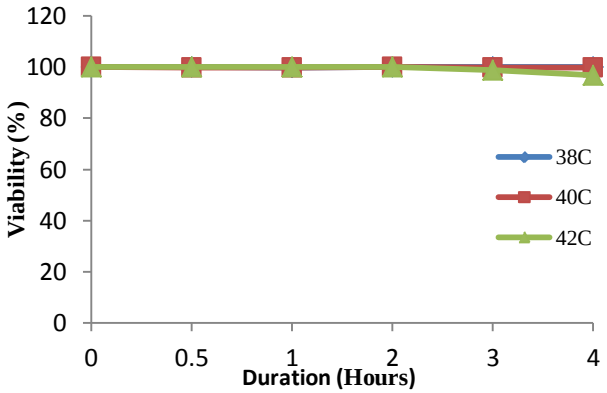


Figure 11: WRL-68 cells after hyperthermic exposure for 4 different temperatures (41, 42, 43 and 44°C), cell viability percentage was determined by methylene blue assay. The data are presented as mean ± SD (SD was within 5% of the mean) from one independent experiment in triplicate.

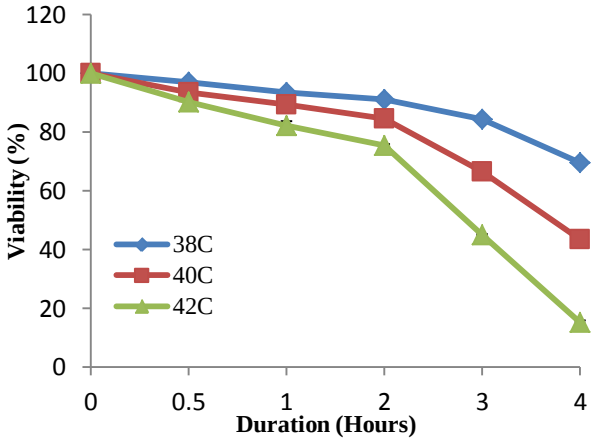


Figure 12: MDA-MB 231 cells after hyperthermic exposure for 4 different temperatures (41, 42, 43 and 44°C), cell viability percentage was determined by methylene blue assay. The data are presented as mean ± SD (SD was within 5% of the mean) from one independent experiment in triplicate.

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. 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. Therefore, hyperthermia might cause cancer cells to undergo apoptosis in direct response to heat. In contrast, healthy cells can more easily maintain a normal temperature. Based on Urano *et al*, 1983 [15] 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 MDA-MB 231 cells were sensitive to temperature of 38°C (refer to figure 10 and 12). 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. Acute acidification pH increases the rate of cell death by decrease heat shock protein levels [16].

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REFERENCES

[1] J. van der Zee. (2002). Heating the patient: A promising approach? *Annals of Oncology*:ESMO. 13(8). pp. 1173- 1184.

[2] H. Fukaoa, M. Ikedaa, H.Ichikawaa, H. Inufusa, K.Okada, S. Ueshima and O.Matsuo. (2000). Effect of hyperthermia on the viability and the fibrinolytic potential of human cancer cell lines. *Clinica Chimica Acta*, 296. pp. 17–33.

[3] P. Hohenberger and C. Kettekhack. (1998). Clinical management and current research in isolated limb perfusion for sarcoma and melanoma. *Oncology*. 55(2). pp. 89–102.

[4] W.C. Dewey, D. Thrall and E.L. Gilette. (1977). Hyperthermia and radiation—a selective thermal effect on chronically hypoxic tumor cells in vivo. *Int J Radiat Oncol Biol Phys*. 2. pp. 99–103.

[5] C.C. Morris, R.Myers and S.B. Field. (1977). The response of the rat tail to hyperthermia. *The British journal of radiology*. 50(596). pp. 576-580.

[6] R.A. Omar and K.W. Lanks. (1984). Heat shock protein synthesis and cell survival in clones of normal and simian virus 40-transformed mouse embryo cells. *Cancer Research*. 44(9). pp. 3976-3982.

[7] M. Watanabe and K. Suzuki. (1989). Heat sensitivity of human cancer cells and abnormal expression of heat shock protein 70. *Japan Journal of Cancer Clinics*. 35(13). pp. 1512-1516.

[8] M.J. Edwards. (1998). Apoptosis, the heat shock response, hyperthermia, birth defects, disease and cancer. *Where are the common links? Cell Stress &Chaperones*, 3(4). pp. 213-220.

[9] C. Jolly and R.I. Morimoto. (2000). Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *Journal of the National Cancer Institute*. 92(19). pp. 1564-1572.

[10] R.S. Williams, J.A. Thomas and M. Fina, *et al*. (1993). Human heat shock protein 70 (hsp70) protects murine cells from injury during metabolic stress. *The Journal of Clinical Investigation*. 92(1). pp. 503-508.

[11] S. Rajdev and F.R. Sharp. (2000). Stress proteins as molecular markers of neurotoxicity. *Toxicologic Patho*. 28(1). pp. 105-112.

[12] M.C. Alley, D.A. Scudiero, A. Monks, M.L. Hursey, M.J. Czerwinski, D.L. Fine, B.J. Abbott, J.G. Mayo, R.H. Shoemaker and M.R. Boyd. (1988). Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res*. 48. pp. 589–601.

[13] G.J. Finlay, B.C. Baguley and W.R. Wilson. (1984). A semiautomated microculture method for investigating growth effects of cytotoxic compounds on exponentially growing carcinoma cells. *Anal. Biochem*. 139. pp. 272–277.

[14] R.J. Ellis and S.M. van der Vies. (1991). Molecular chaperones. *Ann Rev Biochem*. 60. pp. 321–347.

[15] M. Urano, L. Rice, and R. Epstein, *et al.* (1983). Effect of whole-body hyperthermia on cell survival, metastasis frequency, and host immunity in moderately and weakly immunogenic murine tumors. *Cancer Research*. 43(3). pp. 1039-1043.

[16] J.S. Han, C.W. Storck and P.R. Wachsberger, *et al.* (2002). Acute extracellular acidification increases nuclear associated protein levels in human melanoma cells during 42 degrees C hyperthermia and enhances cell killing. *International Journal of Hyperthermia: North American Hyperthermia Group*. 18(5). pp. 404-415.