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Research Article

HYPERTHERMIA EFFECT ON HUMAN NORMAL BREAST (MCF-10A) AND CANCER (MDA-MB 231 AND MCF-7) CELLS

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ABSTRACT

Objective: In this study, the hyperthermia effect on the viability of human normal breast (MCF-10A) and cancer (MDA-MB 231 and MCF-7) cells was evaluated by MTT assay.

Methods: Cells were exposed to heat at 38ºC, 39ºC, 40ºC, 41ºC, 42ºC, 43ºC, and 44ºC for five different durations of heat exposure (0.5, 1, 2, 3, and 4 h). Breakpoint temperatures of MCF-10A, MDA-MB 231, and MCF-7 were determined using cumulative equivalent 43°C (CEM_{43}) model. This model was first time used to calculate thermal isoeffect dose (TID) for MCF-10A, MDA-MB 231, and MCF-7.

Results: MCF-10A started to die at 42°C for 3 h while MDA-MB 231 and MCF-7 need a temperature of 38°C for 0.5 h; thus, they were identified as the threshold temperatures in CEM_{43} model. Furthermore, the effect of "43°C incubator 2 h" had similar total thermal dose as "44°C incubator 0.5 h" for MDA-MB 231 and MCF-7. In addition, "43°C incubator 3 h" effect had also almost the same thermal dose as "44°C incubator 1 h" for MDA-MB 231 and MCF-7.

Conclusion: A better understanding of the significant correlations between CEM_{43} and response parameters in clinical trials could be useful to treat breast cancer patients.

Keywords: Hyperthermia, MCF-10A, MDA-MB 231, MCF-7, cumulative equivalent 43°C model, Thermal isoeffect dose

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INTRODUCTION

Breast cancer is a major global health problem and the most common invasive cancer in women of all ethnic backgrounds. Worldwide, estimated 1.6 million new cases are diagnosed for each year [1, 2]. There are many attempts with a multitude of novel therapeutic concepts although the conventional methods based on surgery, chemotherapy, radiotherapy, or their combinations steadily develop [3].

Hyperthermia among them has attracted significant attention and already entered clinical practice as an adjuvant to chemotherapy and radiotherapy [4]. It is used to raise the temperature of a region of the body affected by cancer with minimal or no damaging healthy tissues [5]. Thermal chemosensitization and thermal radiosensitization effects have been observed both *in vivo* and in *in vitro* cell culture experiments [6]. At least 18 randomized studies have demonstrated that the synergistic effects of combining hyperthermia with either chemotherapy or radiotherapy or both to achieve better therapeutic effects [5]. This was demonstrated for the breast, cervix, head and neck, pancreas, bladder, esophagus, prostate, lung, vulva/vagina cancers, and for melanoma. Hyperthermia has shown great potential in overcoming multidrug resistant (e.g. doxorubicin) which may result in the accumulation of chemotherapy agents within the target cells [6].

Rolf (2008) observed that synergism as a continuous change with increasing the rate temperatures at which cells are killed by the drug [7]. It is generally accepted that when temperatures are raised from 37ºC to over 40ºC, most alkylating agents (e.g., cyclophosphamide and ifosfamide), platinum compounds, and nitrosoureas (bis-chloroethylnitrosourea, and 1-2-chloroethyl-3-cyclohexyl-1-nitrosourea) are linearly enhanced in their cytotoxic effect. On other the hand, threshold temperatures for the interaction with heat at or near 42.5ºC have a synergistic effect with doxorubicin or bleomycin meanwhile most antimetabolites (e.g. 5-fluorodeoxyuridine and methotrexate), vinca alkaloids, or taxanes show independent action [7, 8].

Thus, thermal isoeffect dose (TID) is important because it helps to predict the outcome *in vitro* for a given heat dose. It can be applied to sensitize phenomena with cytostatic drugs, anticancer agents, and radiation therapy to improve better outcome in breast cancer treatment. The TID for induction of cell death was found to be closely related to the amount of energy required to inactivate proteins and enzymes [9]. Although the Arrhenius analysis could be used to calculate the inactivation energy, it is hard to compare two different time-temperature combinations in that plot. Therefore, Sapareto and Dewey [10] described the term "TID" (meaning two different time-temperature combinations produced the same cell killing effect) for comparing different time-temperature combinations. Calculation of the thermal dose applied in hyperthermia has been successfully integrated into the concept of a TID during a certain duration exposure at a given temperature.

Treatment outcome varied greatly between different types of cell lines although the same or different settings of hyperthermia used. For example, the proliferation of human osteosarcoma cells was inhibited by hyperthermia treatment at 42°C whereas heat shock at 44°C inhibited proliferation significantly in normal fibroblasts cells [9]. Therefore, different mechanisms were involved in heat shock-induced cell death among normal cell and cancer cell [5]. According to Omar and Lanks [11] investigated that cancer cells are more susceptible to killing by heat than normal cells after the hyperthermia treatment (43°C–45°C).

In the present study, it was aimed to investigate the percentage viability evaluated by MTT assay with time of heat treatments in breast carcinoma (MDA-MB 231 and MCF-7) and normal breast (MCF-10A) cell lines using seven different temperatures (38°C, 39°C, 40°C, 41°C, 42°C, 43°C, and 44°C) for five different durations of heat exposure (0.5, 1, 2, 3, and 4 h). Moreover, breakpoint temperatures of MCF-10A, MDA-MB 231, and MCF-7 cells were determined using cumulative equivalent 43°C (CEM_{43}) model. This model was first time used to calculate TID for MCF-10A, MDA-MB 231, and MCF-7 cell lines.

METHODS

Cells

The human breast cancer lines (MDA-MB 231 and MCF-7) were cultured in Dulbecco's modified Eagle's medium (DMEM) 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. DMEM/Ham's Nutrient Mixture F-12 was supplemented with 1% penicillin/streptomycin, 5% horse serum 1 ng/mL of cholera toxin, 10 µg/ml of human insulin, 10 ng/ml of epidermal growth factor, and 0.5 µg/ml of hydrocortisone which was used to culture MCF-10A cells. Cells were maintained in a 5% CO_2 humidified incubator at 37°C.

Hyperthermic exposure

MCF-10A, MDA-MB 231, and MCF-7 cells, 1×10^4 cells/well in 200 µl culture medium, were seeded in each well of 96-well plates and precultured overnight incubation at 37°C. Then, hyperthermic exposure was performed by placing culture plates in an incubator maintained at 38ºC, 39ºC, 40ºC, 41°C, 42°C, 43°C, and 44°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 h of hyperthermic exposure for each temperature.

Observation under phase contrast microscope

After hyperthermia treatment, MCF-10A, MDA-MB 231, and MCF-7 cells were observed using a phase contrast microscope (Nikon Ti Eclipse). The photographs were taken at ×10/0.03 magnification.

MTT assay

MTT assay [12, 13] is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple color. The culture medium was removed from the culture plates after appropriate treatment and added with 200 µl of fresh culture medium and 20 µl of MTT media. The well plates were incubated for 4 h at 37°C. The mixture was aspirated slowly from the wells. Then, a volume of 200 µl of isopropanol was added to each well. Well plates were kept in dark for 15 minutes. Finally, absorbance at 570 nm in each well was measured using an ELISA microplate reader (BioTek Instrument). This value was then background subtracted (from media only wells) at 650 nm 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.

Cell viability percentage was calculated using the following equation as below:

% cell viability = (Optical density of hyperthermia treated cells/Optical density of untreated cells) ×100%.

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 one experiment performed in triplicates. Significance testing was performed where indicated using one-factor analysis of variance. The differences were evaluated statistically significant at $p<0.05$.

Calculation of thermal isoeffect dose

The concept of a TID has been successfully integrated from the calculation of the thermal dose during a certain duration of heat exposure at a given temperature. This relationship can be expressed mathematically by the following isoeffect equation:

$$t_1=t_2 * R\,(T_2-T_1) \quad (1)$$

where T_1 and T_2 are two different temperatures of treatment and t_1 and t_2 are the duration exposure of heat at temperature T_1 & T_2, respectively. The shape of the dose-response curve is defined by empirical value, R. R is usually assumed as 0.5 above 43 °C and 0.25 below 43°C by Sapareto and Dewey [10]. Heating time periods at different temperatures are converted into equivalent heating minutes at 43°C which can be explained using the TID concept. From equation (1), if set T1 = 43°C and allow T_2 to be varied during heat treatment, get which is the widely used cumulative equivalent minutes 43°C model.

$$CEM_{43} = \int R\left(43 - T\left[t\right]\right)dt \quad (2)$$

RESULTS AND DISCUSSION

Heat shock treatment on the viability of MCF-10A, MDA-MB 231 and MCF-7 cell lines

In the present study, hyperthermia-induced cytotoxicity was assessed using MTT assay which confirmed that hyperthermia stress greatly decreased cell viability of MDA-MB 231 and MCF-7 cells with increasing temperature (from 37°C to 44°C) and duration of heat exposure (from 1 to 4 h) while MCF-10A cells were maintained the same number as before hyperthermia treatment (as control) from 37°C to 42° C for 2 h. There was a significant statistical difference when the percentage viability of MCF-10A, MDA-MB 231, and MCF-7 cells after the treatment was compared with the control (100%) ($p<0.05$) (Table 1). However, there was a mild decrease in the sum of MCF-10A viable cells as the duration of heat exposure increased from 2 to 3 h at the temperature of 42°C. Based on Table 1, MCF-10A cell viability did not cause any decrease compared to control (100%) from 37°C to 40°C for 0.5 to 4 h and 42°C for 0.5 to 2 h.

Treatment outcome varied hugely between different types of cell lines although the same settings of hyperthermia used. Furthermore, small changes in hyperthermia settings could influence greatly difference in the final treatment outcome. Therefore, the viability of normal and cancer cells is highly influenced by the temperature and duration exposure of heat (hyperthermia settings). For example, induction of apoptosis for human fibrous histiocytoma cells was inhibited hyperthermia treatment at 43°C for 1 h, a temperature lower than that was not able to induce apoptosis although increase the duration of heat exposure. However, Yonezawa *et al*. observed that necrotic death occurred at temperature of 46°C for 1 h where cell death was passive and involved lysis formation from the damaged cell and the release of its cellular content to the surrounding environment [14]. In contrast, heat shock at 44°C inhibited induction of apoptosis significantly in normal fibroblasts cells [9]. Therefore, different mechanisms were involved in heat shock-induced cell death among normal cell and cancer cells [5].

In this study, MDA-MB 231 and MCF-7 cells were started to die at temperature of 38°C for 0.5 h, whereas MCF-10A cell line was started to show cytotoxicity at 42°C for 3 h of hyperthermic exposure. It was reasonable because MDA-MB 231 and MCF-7 cell lines sensitive to heat; therefore, the facilitating effect of hyperthermia to cancer cell was obvious. This was due to MDA-MB 231 and MCF-7 cells did not have a defense system against heat, as did MCF-10A cells [15]. MCF-10A might express heat shock-induced proteins to reactivate denatured proteins; degrade abnormally structured proteins; and inhibit secretion of abnormal proteins and assisting the transfer of secretory proteins by blockage of folding [16]. In addition, Hsp70 induction by hyperthermia treatment in cancer cells is lower than those in normal cells [17].

Table 1: Cell viability percentage of MCF-10A, MDA-MB 231, and MCF-7 following heat treatment[a]

Cells	Time (hours)	38°C	39°C	40°C	41°C	42°C	43°C	44°C
MCF-10A	0	100±0.086[b]	99.916±0.251[b]	99.815±0.185[b]	100±0.172[b]	100±0[b]	100±0.504[b]	100±0.095[b]
	0.5	99.914±0.086	99.916±0.168	99.908±0.277	99.914±0.172	100±0*	99.496±0.504	97.533±0.19
	1	99.829±0.342*	99.916±0.168	100±0.185*	99.828±0.172*	100±0.19	98.69±0.604	93.074±0.38
	2	99.914±0.171	99.916±0.251	99.908±0.185*	99.914±0.172	100±0*	96.979±0.705	89.943±0.095*
	3	100±0.086	99.916±0.168	99.908±0.185	99.914±0.086	100±0.095	95.77±0.302	87.192±0.285*
	4	99.914±0.086	99.916±0.168	100±0.092	99.914±0.258	100±0*	91.339±0.403	81.12±0.19*
MDA-MB 231	0	100±1.221[b]	100±0.592[b]	100±0.548[b]	100±1.261[b]	100±1.469[b]	100±0.412[b]	100±0.105[b]
	0.5	97.447±0.888*	95.008±1.110	94.189±1.316	93.277±0.840	91.081±1.154	78.991±0.515	67.613±0.210
	1	94.229±1.221	92.470±0.761*	90.351±1.206*	87.605±1.261*	83.736±1.889*	62.822±0.412*	47.950±0.210*
	2	92.231±2.109*	89.932±0.761*	85.417±2.083*	79.892±3.466*	76.390±1.889*	52.317±1.339*	35.331±0.315*
	3	84.795±1.554*	79.019±1.523*	67.325±4.605*	55.042±1.786*	46.065±3.148*	27.909±0.515*	10.410±0.210*
	4	69.256±2.331*	55.076±0.592*	43.860±4.057*	29.412±3.027*	16.159±2.833*	9.784±0.515*	0.421±0.315*
MCF-7	0	100±1.378[b]	100±0.347[b]	100±2.286[b]	100±1.038[b]	100±3.769[b]	100±0.418[b]	100±0.815[b]
	0.5	98.163±0.459	96.412±0.347	94.857±1.029	95.386±0.692	92.108±1.413	79.937±0.418	68.685±0.931
	1	96.326±0.689*	94.290±0.694*	91.886±2.286*	89.043±2.653	86.219±3.534*	64.159±0.627*	49.942±3.492*
	2	93.226±1.378*	90.509±1.505*	86.629±2.857*	82.122±2.191*	80.448±1.885*	53.083±1.149*	43.772±2.678*
	3	88.634±2.526*	80.093±1.157	68.914±2.286*	58.362±2.307	49.234±3.062*	29.467±0.522*	15.483±2.678*
	4	71.642±3.674*	57.639±2.894*	44.686±2.626*	31.949±2.884*	19.317±3.887*	11.494±0.418*	1.513±1.048*

[a]Values represent mean±SD of three measurements from one independent experiment, [b]The viability of MCF-10A, MDA-MB 231 and MCF-7 cells at 37°C as control, *p<0.05. SD: Standard deviation

Therefore, MCF-10A cells were survived in undesirable circumstances such as at 42°C incubator hyperthermia for 2 h. However, cell death of MCF-10A cells occurred when temperature increased to 42°C (>2 h of heat exposure). MCF-10A, MDA-MB 231, and MCF-7 cells might die exponentially by induction of apoptosis where cell death was active. 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 an extreme condition such as hyperthermia treatment on MDA-MB 231 and MCF-7 cells at a temperature of 44°C for 4 h. In this study, normal cell line might die exponentially by induction of apoptosis but they might not reach until necrosis. Based on Gabai and Kabakov study [18], cytoskeleton damages and impairment of DNA repair system caused by hyperthermia treatment most likely occur during mitosis; therefore, MDA-MB 231 and MCF-7 (cancer) cells were more susceptible to heat than MCF-10A (normal) cell as cancer cells underwent faster cell division than normal cells. According to Watanabe and Suzuki (1989) [19], normal cells have reduced heat sensitivities 5–6 times at growing condition while cancer did not.

Although cancer therapies such as chemotherapy and radiotherapy can induce tumor cell apoptosis, physiological stress conditions such as growth factor, starvation, hypoxia, or heat can also be equally effective to DNA damaging treatment [20]. Abnormalities of these environmental factors affect tumor cell proliferation and response to antitumor therapy. Tumor 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 study [21], tumor 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 and MCF-7 cells were sensitive to the temperature of 38°C. Besides that, as shown in Song *et al*.'s study [22], rat muscle and skin have a greatly enhanced blood flow at temperatures above 42°C while tumors 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 resultant accumulation of lactic acid [23]. Acute acidification pH increases the rate of cell death by decrease heat shock protein levels [24].

CONCLUSION

The temperature of 42°C for 2 h was chosen as the most suitable temperature and duration of heat exposure to kill cancer cells (MDA-MB 231 and MCF-7) without damaging normal cells (MCF-10A).

Calculation of thermal isoeffect dose

Cell death of MCF-10A, MDA-MB 231, and MCF-7 cell lines from 37°C to 44°C for 0.5 h up to 4 h was studied. When exponentially growing cultured cells (MCF-10A, MDA-MB 231, and MCF-7) were exposed to a defined temperature between 37°C and 44°C of heat exposure, a dose-effect curve was defined by plotting the rate of cell viability against the duration of hyperthermia. The survival curves of MDA-MB 231 and MCF-7 cell lines show a linear growth arrest at the beginning of heat exposure which reflecting reversible and nonlethal heat damage and then, followed by exponential cell death. This is typical "shoulder" which reflects a two-step process of cell killing. However, after increasing the temperature to 43°C, more cells were killed during the same period of heat exposure. Therefore, it was concluded that once the hyperthermia treatment starts to show cytotoxicity, the rate of cell death, which is exponential with exposure duration, is dependent on the temperature of exposure [25].

Arrhenius plot is hard to use for compare two different time-temperature combinations. Thus, the term "TID" (meaning two different time-temperature combinations produced the same cell killing effect) was proposed by Sapareto and Dewey for comparing different time-temperature combinations [10]. The thermal dose can be described as the cumulative equivalent minutes at 43°C for any hyperthermia treatment.

In this study, MDA-MB 231 and MCF-7 cells need temperature of 38°C for 0.5 h to induce apoptosis; therefore, it was identified as the threshold temperature in CEM_{43} model. For MCF-10A cell line, it was clearly indicated that a 42°C hyperthermia treatment for 3 h started to show very little cytotoxicity (Table 1), so the breakpoint for MCF-10A was at temperature of 42°C for 3 h.

Table 2 shows calculated CEM_{43} for several different types of hyperthermia treatment based on the survival curves and Equation 2. As a result, the thermal dose (killing effect) of MCF-10A was lower than MDA-MB 231 and MCF-7 although same settings of hyperthermia used. Effect of "43°C incubator 2 h" had similar total thermal dose as "44°C incubator 0.5 h" for MDA-MB 231 and MCF-7 cells. In addition, "43°C incubator 3 h" effect had also almost the same thermal dose as "44°C incubator 1 h" for MDA-MB 231 and MCF-7 cell lines. This was defined as TID.

The thermal energy dose was required to induce exponential cell death (inactivation energy) which is closely correlated to that required for cellular proteins and enzymes denaturation [10]. It can be combined with cytostatic drugs (e.g. 5-fluorouracil, melphalan, and paclitaxel),

Table 2: Equivalent heating minutes at 43°C using thermal isoeffective dose concept

Cell	Treatment	CEM_{43} (min)
MCF-10A	43°C incubator 0.5 h	0.065
	43°C incubator 1 h	0.331
	43°C incubator 2 h	1.675
	43°C incubator 3 h	8.486
	43°C incubator 4 h	19.102
MCF-10A	44°C incubator 0.5 h	0.659
	44°C incubator 1 h	2.680
	44°C incubator 2 h	10.216
	44°C incubator 3 h	34.622
	44°C incubator 4 h	69.030
MDA-MB 231	43°C incubator 0.5 h	52.431
	43°C incubator 1 h	63.931
	43°C incubator 2 h	95.051
	43°C incubator 3 h	141.318
	43°C incubator 4 h	210.107
MDA-MB 231	44°C incubator 0.5 h	93.009
	44°C incubator 1 h	196.604
	44°C incubator 2 h	329.430
	44°C incubator 3 h	490.662
	44°C incubator 4 h	685.129
MCF-7	43°C incubator 0.5 h	54.478
	43°C incubator 1 h	69.021
	43°C incubator 2 h	110.788
	43°C incubator 3 h	177.830
	43°C incubator 4 h	285.441
MCF-7	44°C incubator 0.5 h	96.340
	44°C incubator 1 h	211.158
	44°C incubator 2 h	380.008
	44°C incubator 3 h	607.891
	44°C incubator 4 h	911.654

Calculated CEM_{43} for different types of hyperthermia treatment for MCF-10A, MDA-MB 231, and MCF-7 cell lines

anticancer agents, radiation, gene, and immunotherapy to achieve better outcome in breast cancer treatment. For example, the use of simultaneous application of weekly cisplatin and regional hyperthermia resulted in a 50% response rate. However, the response rate was about 15% without regional hyperthermia for patients with locoregional advanced cervix carcinoma in a clinical trial. A better understanding of the significant correlations between CEM_{43} and response parameters in clinical trials could useful to treat breast cancer patients.

AUTHOR'S CONTRIBUTIONS

AE designed the research, performed the experiments, analyzed data, and wrote the paper. NJBMA provided the MCF-10A cells. SH contributed critical comments and suggestions. All authors read and approved the final manuscript.

CONFLICTS OF INTERESTS

All authors of this publication declare that there are no conflicts of interest in publishing this research article.

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