

CRITICAL PARAMETERS INFLUENCING HYPERTHERMIA-INDUCED CELL DEATH IN MYELOGENOUS LEUKEMIA CELL LINE (M-NFS-60)

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Hyperthermia is sufficient to induce cell death in many cancer cells. Here we investigated the effect of some parameters that modulate hyperthermia-induced cell death in myelogenous leukemia cell line (M-NFS-60). Trypan blue exclusion method was used to measure M-NSF-60 cell viability after heat exposure to different temperatures (39, 41 and 43 °C) for different periods of time (15, 30 and 60 min). Induced apoptotic cell death after heat exposure was investigated by western blot analysis for the expression level of active caspase-3 protein. The results of this work revealed that M-NFS-60 cells respond to hyperthermia in a graded fashion depend on heat load, time exposure and cell density. Heat exposure at 39 °C and 41 °C for 15, 30 and 60 min and at 43 °C for 15 min caused cell growth arrest, however, at 43 °C for 30 and 60 min it gradually inhibited the growth of M-NFS-60 cells. These data suggest that heat stress at 43 °C at least for 30 min and more has therapeutic efficiency in leukemic cells. Finally, we determined heat load, time exposure and cell density as critical parameters influenced cytotoxic effect of hyperthermia on leukemic cells.

Keywords: Hyperthermia; heat load; time exposure; cell density; myelogenous leukemia, M-NFS-60.

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INTRODUCTION

Cancer is a severe disease and considered to be one of the main leading causes of death in the world. The increase of the individuals' age and the exposure to carcinogenic agents (e.g. tobacco smoke, benzene, and radiation) may be the reasons of the increase of cancer cases [1]. Because cancer refers to a group of diseases, many treatment options exist including surgery, chemotherapy, radiation therapy, hormonal therapy, immunotherapy, and targeted therapy [2]. Removing the solid tumor by surgery is the most effective cancer treatment. However, total remission of cancer can't occur by surgery alone [3,4]. Conventional cancer treatments such as chemotherapy and radiotherapy do not effectively distinguish between rapidly dividing normal cells (e.g., bone marrow,

gastrointestinal tract and those functioning in hair growth) and neoplastic cells, thus leading to several toxic side effects [5]. So there was an urgent need to find other alternatives to current cancer treatments more specific to increase patient's quality of life.

Hyperthermia (Ht, thermal therapy, thermotherapy) is an old technique and currently used as a cancer therapy [6,7]. Over 5000 years ago, ancient Egyptians used heat to treat several diseases. As recorded in the Egyptian papyrus, they used heated stick to cauterize the breast tumor. Also, Hippocrates treated superficial tumors by heated metals [8]. In the field of oncology, hyperthermia is recognized as an artificial method of increasing the body tissue temperature by delivering heat obtained from external sources to destroy neoplastic cells. Hyperthermia is mostly identified with a range of temperatures from 40 to 48 °C which maintained at a treated site for a period of time. Methods used to apply heat to the malignant tissues include hot water blankets, pyrogens (for example, a mixture of bacterial toxins), resistive wire implants, perfusion heating, thermal chambers, ferromagnetic seeds, infrared radiators and nanoparticles [9-11].

Hyperthermia is sufficient to cause direct damage to cancer cells with usually minimal or no injury to normal cells. This concept is based on the higher sensitivity of neoplastic cells to heat shock-induced cell death when compared to normal cells [12-14]. Hyperthermia is generally well tolerated if the temperature does not exceed 44 °C. Higher temperature can lead to blistering, burns or pain. In case of perfusion techniques there may appear swelling of the heated tissue, ischaemia due to blood clots or bleeding. On the whole, hyperthermia side effects are transient [15-17].

Many different types of cancer cells die by hyperthermia alone or as an adjuvant therapy [18-23]; what remains in question are the parameters that trigger the cell death. In this communication, we wanted to investigate some critical parameters that affect the hyperthermia-induced cell death in myelogenous leukemia cell line (NFS-60).

MATERIALS AND METHODS

Chemicals and reagents

RPMI (with L-glutamine) growth medium, fetal bovine serum (FBS), anti biotic mix (10.000 U penicillin/ml, and 10.000 U streptomycin/ml), and phosphate buffered saline (PBS) were purchased from Gibco (Invitrogen, CA. USA). Trypan blue dye, digitonin, sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, B mercaptoethanol,

bromophenol blue, ethylene diamine tetra-acetic acid (EDTA), ethylene glycol tetra-acetic acid (EGTA), phenyl methyl sulfonyl fluoride (PMSF), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), tris-base, tris-HCl, nonidet-P40, tritonX-100, Na dioxycholate, sodium chloride (NaCl), glycerol, glycine, ethanol, methanol, propanol, tween-20, bovine serum albumin (BSA), skim milk (fat free milk powder) and goat anti mouse and mouse anti goat IgG- horseradish peroxidase (HRP) conjugated antibodies were purchased from Sigma (St. Louis, MO, USA). Mouse anti caspase-3 IgG antibody and Goat anti β -actin IgG antibody were purchased from Santa Cruz Biotechnology. Enhanced chemiluminescent (ECL) substrate was purchased from (Thermo Scientific, Rockford, USA). Cell culture dishes and glassware were purchased from high quality local suppliers.

Cell culture

M-NFS-60 cell line (myelogenous leukemia cells) was purchased from VacSera Egypt, Alagoza, Algeza Egypt. Cells were cultured in RPMI-1640 (with L-glutamine) growth medium supplemented with 10% (v/v) FBS, and 1% antibiotic mix (10.000 U penicillin/ml, and 10.000 U streptomycin/ml) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

Cell treatment

M-NFS-60 cells were exposed to thermal shock in controlled water bath at different temperatures (39, 41 and 43 °C) for 15, 30 and 60 min. Cells were returned to a controlled 37 °C incubator for the desired recovery time. To ensure the temperature accuracy, the temperature was monitored by a thermometer securely inserted in a reference plate similar to the culture plate and both were treated with the same conditions of temperature and time.

Cell viability: trypan blue exclusion method

Cell viability was assessed by trypan blue exclusion method. Viable cell had a clear cytoplasm according to exclusion of the dye whereas dead cell had a blue cytoplasm [24]. In the present study, 100 μ l of cell suspension was simply mixed with 100 μ l of 0.4% trypan blue in a 1.5 ml Eppendorf tube and incubated at room temperature for ~3 min. The mixture was transferred to the edge of the two counting chambers of hemocytometer for allowing the cell suspension to be drawn into the counting chamber by capillary action. Using light microscope, viable (bright) cells were counted in the four large corner counting squares.

Western blot analysis

Heated cells were pelleted by centrifuging at 1,500 rpm for 5 min at room temperature, washed twice with ice-cold PBS and lysed with ice-cold lysis buffer (1% Nonidet-P40, 1% TritonX-100, 0.5% Na dioxycholate, 150 mMNaCl, 5 mM EDTA, 10 mM EGTA, 50mMTris-HCl, 10% SDS and1mM PMSF). The lysates were centrifuged at 2,500 rpm for 10 min at 4 °C to obtain protein extracts. Equivalent protein extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gel. The proteins were then transferred onto nitrocellulose membranes, and probed with primary antibodies (anti-caspase-3 and anti- β -actin, 1:1000, overnight at 4 °C), followed by secondary antibodies (1:10000 for 1 h at room temperature). Detection was performed using ECL substrate. Relative pro- and active caspase 3 levels were carried out by estimation of non-calibrated optical density of each band including actin bands by ImagJ software. The values from three independent experiments were normalized to the corresponding actin levels, each value was divided by the value of control cells (at 37 °C) and expressed as mean \pm SD.

Statistical Analysis

The values of cell viability were presented as relative to unheated control cells (37 °C). Cell viability was considered to be 100% in the control group. Values are presented as mean \pm standard error (SE) of three independent experiments. Statistical significance of differences was analyzed by One-way ANOVA test followed by Newman-keuls post-test. P value $<$ 0.05 was considered significant.

RESULTS

Effects of heat load on the viability of M-NFS-60 cells

M-NFS-60 cells were exposed to increasing heat loads (39, 41 and 43 °C) for different periods of time (15, 30 and 60 min). After 24 & 48 h of heating, the viability of cells was significantly ($p <$ 0.05) decreased by increasing the temperature of heating over the tested time range (15, 30 and 60 min) when compared with unheated control cells (37 °C). Also, there were significant differences ($p <$ 0.05) among the different temperatures over the tested time range, while the difference between 39 and 41 °C for 15 min after 24 h was not significant. For all tested time (15, 30 and 60 min), 39 and 41 °C-heated cells regrew after 24 h of

heating. Also, 43°C-heated cells recovered their proliferative ability after 24 h of heating when heated for 15 min. However, 43 °C-heated cells didn't regrow after 24 h of heating when heated for 30 or 60 min (Figs. 1,2).

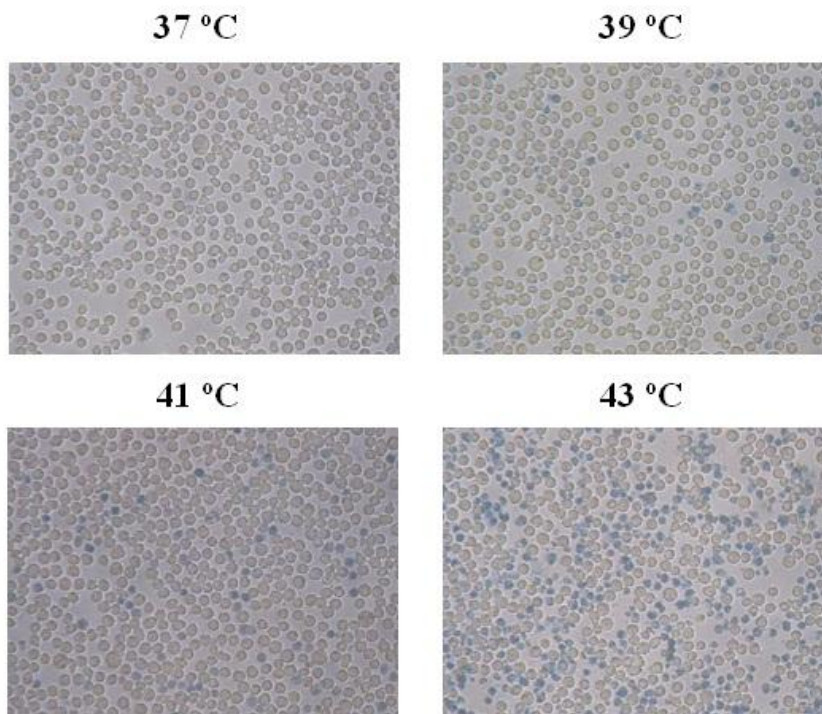


Fig. 1: Photographs showing cell viability using trypan blue exclusion method after heating M-NFS-60 cells at 39, 41 and 43 °C. Viable cells have clear cytoplasm whereas dead cells have blue cytoplasm. Representative of at least three independent experiments, X 400.

As shown in Fig. 3, there was no noticeable change in the expression level of active caspase-3 after heat exposure at 39 or 41 °C when compared with unheated control cells (37 °C). The only obvious change in the level of active caspase-3 was detected after heat exposure at 43°C, indicating apoptotic cell death.

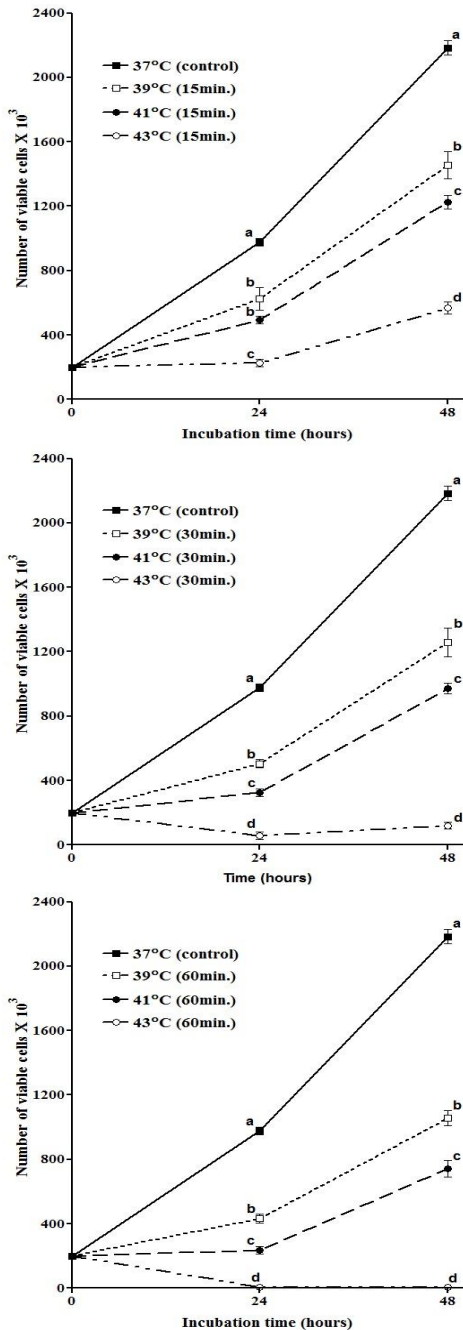


Fig. 2: The effect of heat load on the viability of M-NSF-60 cells. M-NSF-60 cells were heated at 39, 41 and 43 °C for 15, 30 and 60 min. Cell viability was assessed by trypan blue exclusion method after 24 & 48 h incubation. The data are mean values of three independent experiments \pm SE. Values in the same time with unlike superscript letters are significantly different ($p < 0.05$).

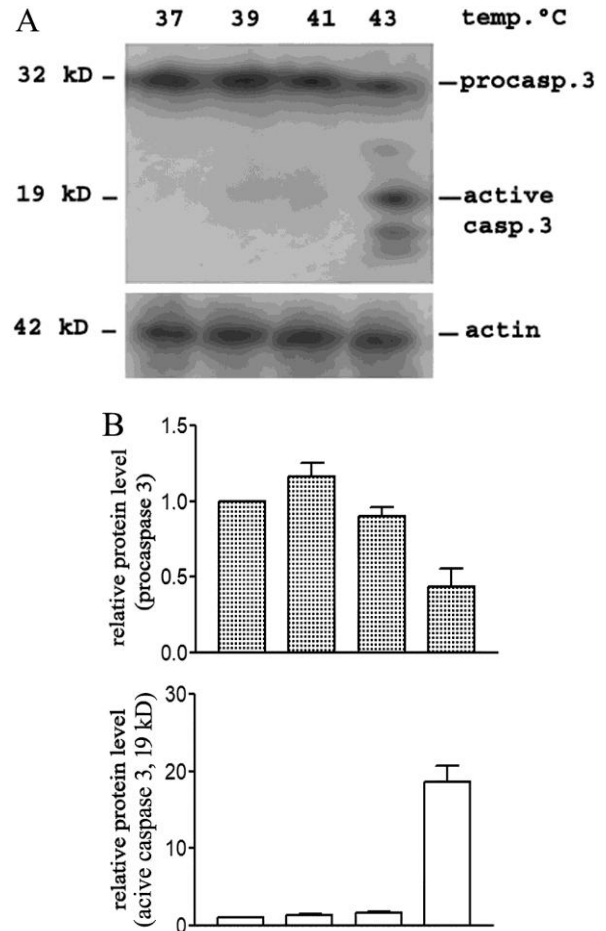


Fig. 3: Western blot showing the levels of pro and active caspase-3 in M-NSF-60 cells after thermal shock. A, a representative western blot after thermal shock at 39, 41 and 43 °C for 30 min. Actin level detection carried out for confirming the equal protein loading. B, the means \pm SD of relative protein levels of pro- and active caspase 3 from three independent experiments and normalized to the corresponding actin levels.

Effects of the time of heat exposure on the viability of M-NFS-60 cells

The data indicated that M-NFS-60 cells were responded to heat exposure over the time range tested (15, 30 and 60 min) with observed decrease in their viability in time-related manner (Fig. 4). By heating at 39 °C, the increase of time exposure from 15 to 30 min decreased the viability of cells but this decrease wasn't significant. However, the increase of time exposure of heating at 39 °C from 15 to 60 min significantly ($p < 0.05$) decreased the viability of cells. By heating at 41 °C, the increase of time exposure from 15 to 30 min or from 15 to 60 min significantly ($p < 0.05$) decreased the viability of cells.

By increasing the time exposure of heating at 43 °C, the viability of cells was decreased. The decrease of cell viability was significant ($p < 0.05$) when comparing each time exposure with the other. However, the difference wasn't significant when comparing between 30 & 60 min for 24 h.

Effects of cell density on the viability of M-NFS-60 cells after heating

As shown in Fig. 5, different densities of M-NFS-60 cells (25, 50, 100 and 200 X 10³ cells/ml) were heated at 39, 41 and 43 °C for 60 min. After incubation for 24 & 48 h of heat loaded cells at 39 and 41 °C, the viability of cells was incrementally decreased by decreasing the density of cells. The decreasing of cell viability was significant ($p < 0.05$) when comparing each cell density with the other. However, the difference wasn't significant when comparing between 25 X 10³ and 50 X 10³ cells/ml after incubation for 24 h.

Also, the positive correlation between cell density and cell viability was detected after 24 & 48 h of heat loaded M-NFS-60 cells at 39, 41 and 43 °C for 15 and 30 min (data, not shown). After 24 & 48 h incubation of heat loading at 43 °C for 60 min, the effect of cell density on cell viability wasn't observed because there was a strong cell death.

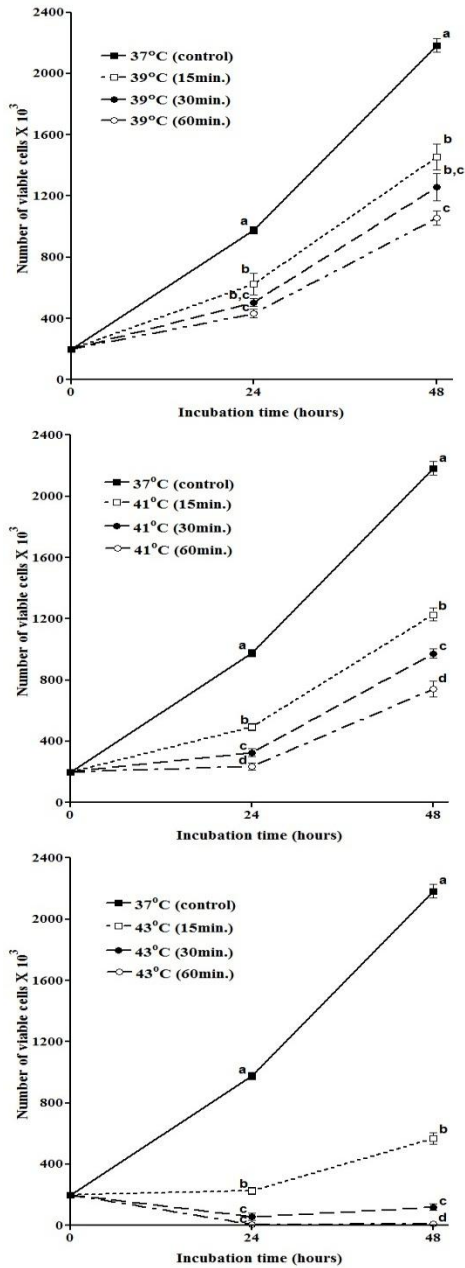


Fig. 4: The effect of the time of heat exposure on the viability of M-NSF-60 cells. M-NFS-60 cells were heated for 15, 30 and 60 min at 39, 41 and 43 °C. Cell viability was assessed by trypan blue exclusion method after 24 & 48 h incubation. The data are mean values of three independent experiments \pm SE. Values in the same time with unlike superscript letters are significantly different ($p < 0.05$).

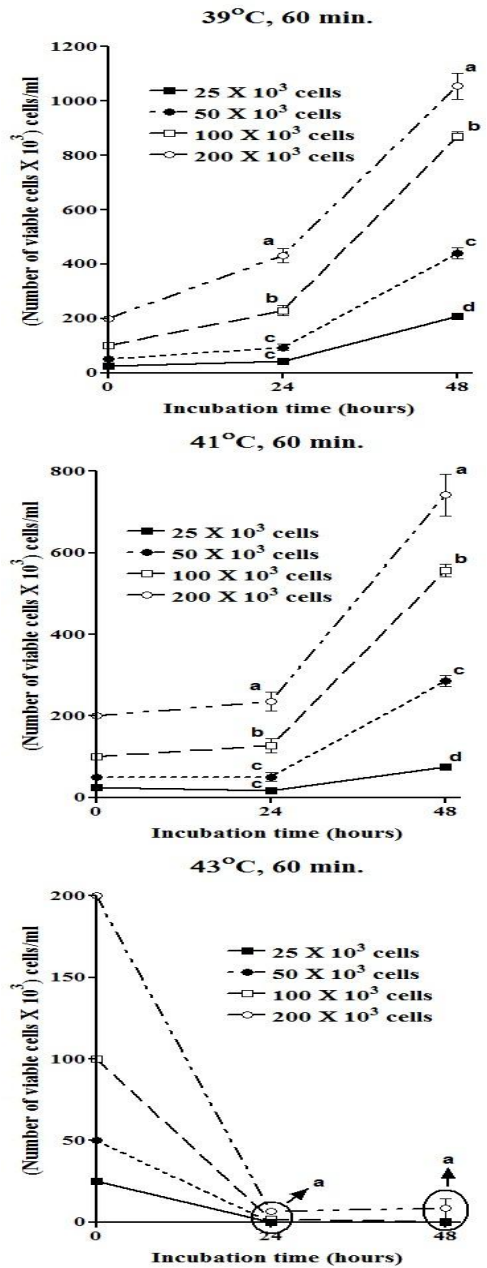


Fig. 5: The effect of cell density on the viability of M-NSF-60 cells after heating. Different densities of M-NFS-60 cells (25, 50, 100 and 200 X 10³ cells/ml) were heated at 39, 41 and 43 °C for 60 min. Cell viability was assessed by trypan blue exclusion method after 24 & 48 h. The data are mean values of three independent experiments \pm SE. Values in the same time with unlike superscript letters are significantly different ($p < 0.05$).

DISCUSSION

Hyperthermia is defined as the application of exogenous heat induction and has been known to inhibit cell growth in different cancer cells [25,26]. Hyperthermia was reported to induce cell death of four cultured human carcinoma cell lines, fibrosarcoma cells (HT-1080), lung adenocarcinoma cells with highly metastatic potential (HAL-8), melanoma cells (Bowes) and osteosarcoma cells (NY) [27]. In the present study, hyperthermia at 39, 41 and 43 °C decreased the proliferative capacity of M-NFS-60 myelogenous leukemia cells (Figs. 1,2). These results are in agreement with those of Speit and Schütz [28] who demonstrated that proliferation of A549 human lung cell line was clearly inhibited at 41 °C and 42 °C. Also, Goliaei *et al.* [29] showed that hyperthermia at 41 °C caused cytotoxicity and growth arrest and reduced clonogenicity in K562 erythroleukemic cell line. Moreover, Yavelsky *et al.* [30] reported that heat treatment showed cytotoxic effect on SKOV3 human ovarian carcinoma cells.

We tested the parameters affecting cell death induced by hyperthermia in M-NFS-60 cells where cells were given increasing heat loads (39, 41 and 43 °C) for different periods of time (15, 30 and 60 min), followed by 24 & 48 h recovery periods at 37 °C. Data from the current study showed that hyperthermia induced cytotoxic effect in M-NFS-60 cells in heat load-dependent manner (Figs. 1,2). This result was come in line with previous result obtained by Pawlik *et al.* [31] who reported that heating non-small cell lung cancer cell line, H1299, at 43.5 and 45 °C caused a statistically significant heat load-dependent decrease in the percentage of viable cells, 24 & 48h after heat treatment. Also, Goliaei *et al.* [29] demonstrated that cell survival of K562 cell line was decreased by increase heating temperature from 41 to 43 °C. In other experiment, viability of Bowes melanoma cells was reduced by 98% at 43°C. By elevating temperature to 45°C their viability was more reduced by 88% [27].

The other parameter considered in the current study was the exposure time of hyperthermia. The exposure of M-NFS-60 cells to 39, 41 or 43 °C for different periods of time (15, 30 and 60 min) caused a cytotoxic effect in time-related manner (Fig. 4). In this topic Speitand Schütz [28] reported that heating at 48 and 43 °C decreased the viability of A549 human lung cell line and bowes human melanoma cell line, respectively, in time-dependent manner. Also, HAL-8 lung adenocarcinoma cells completely survived at 43 °C for 60 min but their

viability was dramatically reduced by 55% with increasing time to 120 min [27]. Moreover, heat treatment alone of human T-lymphoblast cell line (CCRF-CEM) for both 1 and 2 h at 42 °C resulted in 69.7 and 85.9% cell kill, respectively [32].

In an attempt to explore the effect of cell density on cytotoxicity of hyperthermia, M-NFS-60 cells cultured at different densities and exposed to elevated temperatures. Data from the current study showed that elevation of cell density at hyperthermia treatment, at 39 and 41 °C, resulted in increase of cell viability (Fig. 5). To the best of our knowledge, no previous study has investigated the effect of cell density on cytotoxic effect of hyperthermia. However, similar results have been reported using antitumor agents where there was negative correlation between cell density of many cancer cell lines and cytotoxic activity of used drugs [33,34].

The effect of cell density wasn't observed when M-NFS-60 cells were heated at 43 °C for 60 min (Fig. 5). Also, it should be noted that at 39 °C and 41 °C for all tested time and at 43 °C for 15 and 30 min there were a heat load and time-dependent arrest in M-NFS-60 cell growth and cells regrew after 24 h after heat exposure. However, hyperthermia at 43 °C for 60 min totally inhibited the growth of M-NFS-60 cells and cells did not recover their proliferative ability after 24 h from heat exposure (Figs. 2,4). Moreover, the level of active caspase-3 protein was obviously increased at 43 °C (Fig. 3). Therefore, we suggest that M-NFS-60 cells respond to hyperthermia in a graded fashion depend on heat load, time exposure and cell density. Initiating protection mechanism against heat stress might be the reason of cell regrowth after cell growth inhibition by hyperthermia. When intensity of hyperthermia is augmented, the protection mechanism may be insufficient to further protect the cells and the apoptotic program is initiated.

There are many parameters that affect hyperthermia induced cell death in neoplastic cell lines. The present study investigates some critical parameters influencing hyperthermia induced cell death of the hard to treat myelogenous leukemia.

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العوامل الحرارية التي تؤثر في الموت الخلوي المستحث بفرط الحرارة في خط خلايا اللوكيميا النقوية (M-NFS-60)

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فرط الحرارة يكفي للحث على الموت الخلوي في العديد من الخلايا السرطانية. في هذه الدراسة بحثنا تأثير بعض العوامل التي تعدل من موت الخلايا المستحث بواسطة فرط الحرارة في خط الخلايا اللوكيميا النقوي (M-NFS-60). تم استخدام طريقة استبعاد صبغ التريبيان الأزرق لقياس حيوية الخلايا (M-NSF-60) بعد التعرض لدرجات حرارة مختلفة (39 و 41 و 43 درجة مئوية) لفترات زمنية مختلفة (15 و 30 و 60 دقيقة). تم التحقق من الموت

الخلوى المبرمج بعد التعرض للحرارة بواسطة التحليل المناعى للبروتين (الوصمة الغريبة) لمستوى بروتين caspase-3 النشط. أظهرت نتائج هذه الدراسة أن خلايا (M-NFS-60) تستجيب لفرط الحرارة بطريقة متدرجة تعتمد على الحمل الحراري ووقت التعرض للحرارة وكثافة الخلايا. تعرض الخلايا للحرارة عند ٣٩ و ٤١ درجة مئوية لمدة ١٥ و ٣٠ و ٦٠ دقيقة وكذلك تعرضها للحرارة عند ٤٣ درجة مئوية لمدة ١٥ دقيقة تسبب في توقف نمو الخلايا لفترة ثم استعادة القدرة على النمو مرة أخرى، ولكن تعرض الخلايا للحرارة عند ٤٣ درجة مئوية لمدة ٣٠ و ٦٠ دقيقة عطل تدريجيا نمو الخلايا M-NFS-60 مع عدم استعادة القدة على النمو مرة أخرى. هذه البيانات تشير إلى أن تعرض الخلايا للحرارة عند ٤٣ درجة مئوية لمدة ٣٠ دقيقة على الأقل لديه كفاءة علاجية في الخلايا اللوكيميا. أخيرًا ، وجدنا أن الحمل الحراري ووقت التعرض للحرارة وكثافة الخلايا عوامل أساسية تؤثر على موت خلايا اللوكيميا المستحث بواسطة استخدام فرط الحرارة.