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THE EFFECT OF MILD HYPERTHERMIA ON MORPHOLOGY, ULTRASTRUCTURE AND F-ACTIN ORGANIZATION IN HL-60 CELL LINE

WPŁYW ŁAGODNEJ HIPERTERMII NA MORFOLOGIĘ, ULTRASTRUKTURĘ I ORGANIZACJĘ F-AKTYNY W KOMÓRKACH LINII HL-60

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Summary

Introduction. Hyperthermia is a well-established physical stimulus, which is applied as an adjunctive therapy with various cancer treatments, such as radiotherapy and chemotherapy. However, the precise mechanism of heat action at the cellular level remains to be elucidated, and appears to be multi-dimensional. The purpose of the current study was to determine the effect of mild hyperthermia on the actin cytoskeleton in the HL-60 cell line. In addition, the morphological and ultrastructural approaches were used to determine the type of hyperthermia-induced cell death.

Material and methods. All studies were performed using human promyelocytic leukemia cell line (HL-60). Actin filaments were visualized with phalloidin conjugated to Alexa Fluor® 488 using fluorescence microscopy. Morphological and ultrastructural changes in the HL-60 cells were analysed by light and electron microscopy, respectively.

Results. Exposure of HL-60 cells to mild hyperthermia resulted in the reorganization of the actin cytoskeleton and the appearance of characteristic apoptotic features, including cell shrinkage, chromatin condensation and margination. In addition, swollen mitochondria were observed. The morphological and ultrastructural changes increased in severity with an increase in recovery time. Similarly, actin filament remodeling was observed immediately after the heat shock and was more evident 3 and 6 hrs after the treatment. These effects were mainly reflected by a higher definition of the dense cortical F-actin ring as well as the appearance of brightly fluorescent F-actin dots and networks scattered throughout the cytoplasm.

Conclusions. Presented data suggest that actin filament reorganization is involved in the process of apoptosis initiated by mild hyperthermia. Furthermore, the results of our studies showed that the severity of hyperthermia-induced morphological and ultrastructural changes as well as alterations in actin organization depend not only on the temperature treatment but also on the duration of post heat shock recovery.

Streszczenie

Wstęp. Hipertermia jest dynamicznie rozwijającą się metodą lecznia nowotworów, stosowaną w skojarzeniu z radio- i/lub chemioterapią. Precyzyjny mechanizm działania hipertermii na poziomie komórkowym nie został, jak dotąd w pełni poznany i wydaje się on wielowymiarowy. Celem niniejszej pracy była analiza wpływu łagodnej hipertermii na organizację filamentów aktynowych w komórkach linii HL-60. Za zasadną uznano także ocenę zmian morfologicznych i ultrastrukturalnych wywołanych przez hipertermię, celem określenia rodzaju uruchamianej śmierci zachodzącej w badanej linii.

Materiały i metody. Badania przeprowadzono na komórkach linii białaczki promielocytowej HL-60. Filameny aktynowe wyznakowano falloidyną skoniugowaną z Alexa Fluor® 488 i oglądano w mikroskopie fluorescencyjnym. Morfologiczne i ultrastrukturalne zmiany w komórkach oceniono odpowiednio, przy użyciu mikroskopu świetlnego oraz transmisyjnego mikroskopu elektronowego.

W y n i k i . W wyniku ekspozycji komórek HL-60 na podwyższoną temperaturę obserwowano reorganizację cytoszkieletu aktynowego oraz pojawienie się komórek o cechach charakterystycznych dla procesu apoptozy, takich jak obkurcznie cyto- i nukleoplazmy, kondensacja i marginalizacja chromatyny czy obrzmienie mitochondriów. W wyniku rearanżacji, F-aktyna lokalizowała sie głównie w części korowej komórki w formie pierścienia lub w cytoplazmie w postaci wyraźnie wyznakowanych sieci i skupień. Stopień nasilenia zmian w komórkach wzrastał wraz ze wzrostem czasu regeneracji komórek.

W n i o s k i . Uzyskane wyniki pozwalają stwierdzić, że cytoszkielet aktynowy zaangażowany jest w realizację procesu apoptozy indukowanej przez łagodną hipertermię. Ponadto sugerują one, że na wystąpienie zmian w organizacji filamentów aktynowych, jak również zmian w morfologii i ultrastrukturze komórek ma wpływ, nie tylko zastosowany profil temperaturowy, ale również czas regeneracji komórek.

Key words: hyperthermia, actin filaments, HL-60 cell line

Słowa kluczowe: hipertermia, filamenty aktynowe, linia komórkowa HL-60

INTRODUCTION

The term ‘hyperthermia’ refers to raising the temperature of a part of the body or of the whole body above the threshold temperature set at a particular moment by the thermoregulatory system of the organism [1,2]. Hyperthermia has been used for its medical properties since ancient times. Presumably, the oldest written medical report with references to hyperthermia was found in the Egyptian Edwin Smith surgical papyrus, dated around 3000 BC [3]; whereas, the use of hyperthermia for cancer therapy was first reported by Hypocrites in the treatment of breast tumor [4]. Currently, hyperthermia is a well-established physical modality, which is used as an adjunctive therapy with various established cancer treatments, such as radiotherapy and chemotherapy [5].

The scientific rationales for using hyperthermia in cancer treatment are based on the data from in vitro, animal and preclinical studies [3,6]. It has been shown that the alterations in plasma membrane permeability caused by hyperthermia lead to better infiltration and drug absorption by the tumor [7]. It has been also suggested that hyperthermia treatment activates the immune system against the tumor [8]. Moreover, cancer cells are selectively sensitive to heat shock treatment, while the same conditions rarely affect the growth of normal cells. Additionally, an increase in thermosensitivity of tumor cells results in the reduction of tumor blood flow which makes their environment more hypoxic and acidic. Moreover, several studies have shown that hypoxia and particularly acidity enhance the cytotoxicity of hyperthermia [9,10].

The mechanisms of hyperthermia cytotoxicity involve denaturation of cellular proteins and their subsequent aggregation as well as plasma membrane damage, inhibition of DNA repair, and changes in intracellular calcium homeostasis. The heat-induced alterations in cell structure and function lead to cell cycle arrest and cell death [11].

Among the most noteworthy alterations in heat-shocked cells are the induction of heat shock proteins (HSPs) and reorganization of the cytoskeleton [12]. The actin cytoskeleton is involved in the regulation of fundamental cellular processes such as motility, cytokinesis, adhesion and endocytosis [13]. Furthermore, it also participates in signal transduction regulating cell growth, survival, and death. Therefore, the actin cytoskeleton may represent an attractive target for hyperthermic therapy against different types of cancer [13, 14]. However, the mechanism of structural and functional actin remodeling in response to heat shock is not fully understood.

The aim of this study was to determine the effect of mild hyperthermia on actin cytoskeleton organization in the HL-60 cell line, in the context of involvement in cell death process.

MATHERIAL AND METHODS

Cell culture and treatment

The human promyelocytic leukemia cell line HL-60 was purchased from the American Type Culture Collection (ATCC, Manassas, VA; CCL-240). The cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% fetal bovine serum (FBS, PAA) and 10 mg/ml gentamicin (Lonza), at 37ºC in a humidified 5% CO_2 atmosphere. After 48 hr cell culture, HL-60 cells were subjected to heat shock (40ºC, 2 hrs), followed by post-heat shock recovery at 37ºC for 0, 3 and 6 hrs. Control cells were cultured identically without exposure to heat treatment. Cell viability was assessed by the trypan blue dye exclusion method.

Light microscopy studies

For the morphological analysis, HL-60 cells were fixed in 4% paraformaldehyde. After fixation, the cells were incubated with 0.1M glycine solution (Roth) and then the cell suspension was centrifuged onto glass slides. Thereafter, the cells were stained with Mayer's hematoxylin and rinsed under running tap water and dehydrated in a graded series of alcohols and xylenes. The preparations were observed using an Eclipse E800 microscope (Nikon) with NIS-Elements ver. 3.30 image analysis system (Nikon) and CCD camera (DS-5Mc-U1; Nikon).

Transmission electron microscopy studies

For ultrastructural analysis, the cells were fixed with 3.6% glutaraldehyde and then postfixed with 1% osmium tetroxide, dehydrated with graded series of alcohol and acetone, and embedded in Epon 812. The polymerization of the resin was performed at 37°C for 24 hrs, and then at 65°C for 120 hrs. Selected parts of material were cut into ultra-thin sections by using an OmU3 ultramicrotome (Reichert) and then counterstained with uranyl acetate and lead citrate. The material was examined using JEM 100 CX electron microscope (JEOL).

Fluorescence microscopy studies

For fluorescence labeling of actin, the cells were fixed with 4% paraformaldehyde. After fixation, the cells were incubated with 0.1M glycine solution (Roth) and then the cell suspension was centrifuged onto glass slides. The cells were then permeabilized with 0.1% Triton X-100 (Serva Feinbiochemica). To enable visualization of F-actin, the cells were incubated with phalloidin conjugated to Alexa Fluor 488 (Invitrogen, diluted 1:40). The nuclei of the cells were labeled with 4',6-diamidino-2-phenyloindole (DAPI; Sigma-Aldrich). Slides were mounted in Aqua-Poly/Mount (Polysciences) and analysed by using an Eclipse E800 microscope with a Y-FL fluorescence attachment (Nikon), NIS-Elements ver. 3.30 image analysis system (Nikon) and CCD camera (DS-5Mc-U1; Nikon).

Statistical analysis

The non-parametric Mann-Whitney U test was performed to compare the differences between experimental groups. The results were considered statistically significant at $p<0.05$. The GraphPad Prism 5.0 (GraphPad Software) was used for statistical analyses.

RESULTS

Viability of HL-60 cells after mild hyperthermia treatment

The viability was measured by trypan blue dye exclusion assay and expressed as the fraction of viable cells to total cells. The results did not show any differences between the control and these heat-shocked cells which were not subjected to post-heat shock recovery. Following a recovery time (3 and 6 hrs), a slight reduction in cell viability was observed (Fig. 1, Table I). However, statistical analysis showed that these changes were statistically insignificant.

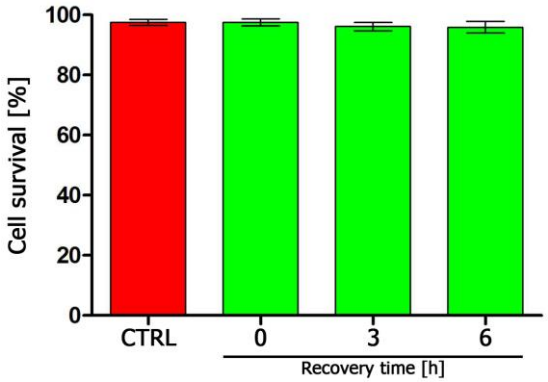

Fig. 1. *The effect of mild hyperthermia on the average percentage of surviving HL-60 cells*

Ryc. 1. *Wpływ łagodnej hipertermii na średni procent przeżywających komórek linii HL-60*

Table I. *The effect of mild hyperthermia on the survival of HL-60 cells. Data in the table presents the mean percentage of viable cells, standard deviation and median*

Tabela I. *Wpływ łagodnej hipertermii na przeżycie komórek linii HL-60. Dane w tabeli przedstawiają średni procent przeżywających komórek, odchylenie standardowe oraz medianę*

	Mean [%]	**Standard deviation**	**Median**
Control	97.5	1.509	98
Hyperthermia 40ºC/2hrs	97.5	1.650	97.5
Hyperthermia 40ºC/2hrs, recovery time 3hrs	96.1	1.912	96.5
Hyperthermia 40ºC/2hrs, recovery time 6hrs	95.9	2.767	96

The effect of mild hyperthermia on the morphology and ultrastructure of HL-60 cells

The evaluation of changes in the morphology and ultrastructure of heat-shocked HL-60 cells was performed. Control HL-60 cells were circular or oval in shape and contained intact nuclei. Only a few cells were morphologically changed (Fig. 2A).

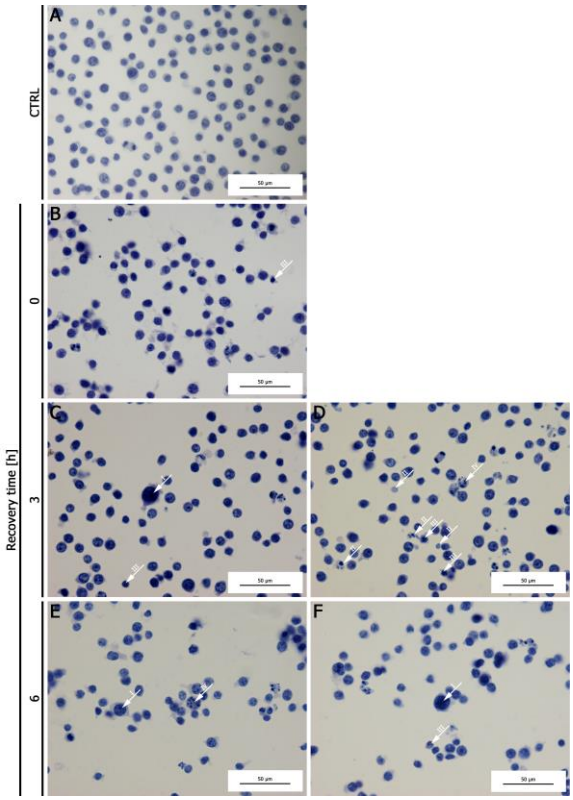

Fig. 2. *The effect of mild hyperthermia on morphology of HL-60 cells, stained with Mayer's hematoxylin; Non-treated cells (A); The HL-60 cells were heat shocked at 40ºC for 2 hrs (B) and returned to 37ºC for: 3 hrs (C,D) or 6 hrs (E,F); The enlarged cells with one big nucleus are seen (C,F arrows I); The shrunken cells with fragmented nuclei (D arrows II) and/or chromatin condensation (B, C, D, F arrows III) can be also observed; The swollen cell with fragmented nucleus is seen (D, E arrows IV)*

Ryc. 2. *Wpływ łagodnej hipertermii na morfologię komórek linii HL-60, barwionych hematoksyliną Mayera; Komórki kontrolne (A); Komórki HL-60 poddano szokowi cieplnemu w temperaturze 40ºC przez 2h (B), po czym ponownie umieszczono w 37ºC na okres 3 (C,D) lub 6h (E,F); Widoczne powiększone komórki z jednym dużym jądrem (C, F strzałki I); Widoczne obkurczone komórki z fragmentację jądra (D strzałki II) i/lub kondensacją chromatyny (B,C,D,F strzałki III) Widoczne również obrzmiałe komórki z fragmentacją jądra (D,E strzałki IV)*

The appearance of HL-60 cells fixed immediately after a mild heat shock (40°C/2hrs) was not significantly different from that of the control (Fig. 2B). However, with increasing recovery time more advanced changes in cell morphology and ultrastructure occurred in the heat-treated cultures. After 3 and 6 hrs of recovery, the shrunken cells with irregular and undulating surface as well as chromatin condensation and marginalization were observed (Fig. 2C,D,F, 3C). The cells with fragmented nuclei were also noticed (Fig. 2D). Additional changes, including swollen mitochondria (Fig. 3C) and vacuolization of the cytoplasm (Fig. 3D) were also detected. Moreover, a few cells with the morphological appearance of mono-nucleated giant cells were seen (Fig. 2C,F).

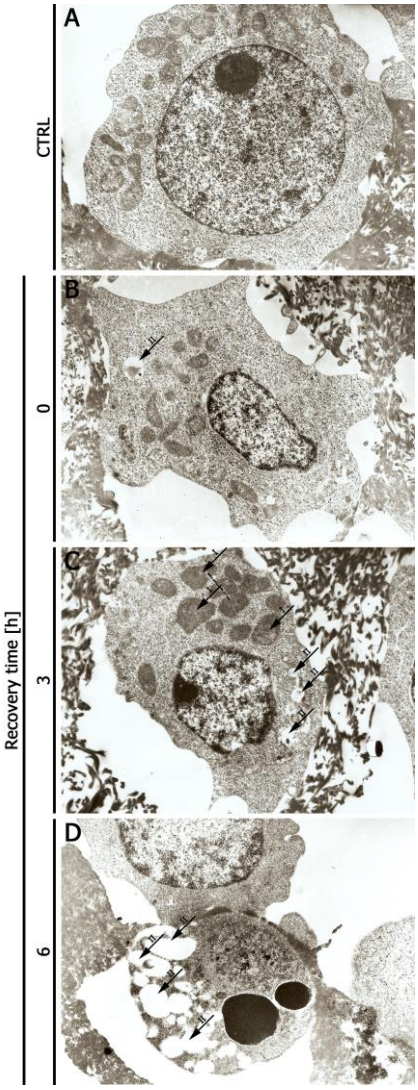

Fig. 3. *The effect mild hyperthermia on the ultrastructure of HL-60 cells; Non-treated cells (A); The HL-60 cells were heat shocked at 40ºC for 2 hrs (B) and returned to 37ºC for: 3 hrs (C) or 6 hrs (D); Visible swollen mitochondria (C arrows I); Cytoplasmic vacuolization is seen (C,D arrows II)*

Ruc. 3. *Wpływ łagodnej hipertermii na ultrastrukturę komórek HL-60; Komórki kontrolne (A); Komórki HL-60 poddano szokowi cieplnemu w temperaturze 40ºC przez 2h (B), po czym ponownie umieszczono w 37ºC na okres 3 (C) lub 6h (D); Widoczne obrzmiałe mitochondria (C strzałki I) oraz wakuolizacja cytoplazmy (C,D strzałki II)*

The effect of mild hyperthermia on F-actin organization

Alexa Fluor 488-phalloidin staining was applied for visualization of changes in filamentous actin organization after treatment of HL-60 cells with mild hyperthermia. Control HL-60 cells displayed a spread F-actin cytoskeleton with well-defined peripheral limits and intact nuclei. Only a few nuclei were morphologically changed (Fig. 4A,A').

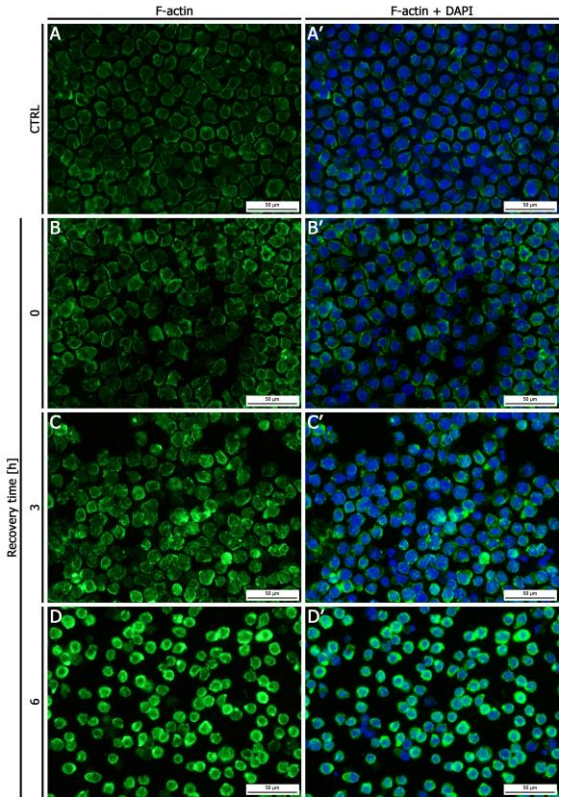

Fig. 4. *The effect of mild hyperthermia on the F-actin organization in the HL-60 cells; F-actin was stained with Alexa Fluor 488 phalloidin, nuclei were counterstained with DAPI; Non-treated cells (A,A'); The HL-60 cells were heat shocked at 40ºC for 2 hrs (B,B') and returned to 37ºC for: 3 hrs (C,C') or 6 hrs (D,D')*

Ryc. 4. *Wpływ łagodnej hipertermii na organizację F-aktyny w komórkach linii HL-60; F-aktynę wyznakowano falloidyną skoniugowaną z Alexa Fluor 488, jądra wybarwiono DAPI; Komórki kontrolne (A,A'); Komórki HL-60 poddano szokowi cieplnemu w temperaturze 40ºC przez 2h (B,B'), po czym ponownie umieszczono w 37ºC na okres 3 (C,C') lub 6h (D,D')*

The structural remodeling of actin was observed immediately after heat shock treatment but was more evident 3 and 6 hrs after the exposure. These effects were reflected mainly by a higher definition of the dense cortical F-actin ring as well as the appearance of bright F-actin clusters and network scattered throughout the cytoplasm (Fig. 4C,D). Moreover, directly after heating, the cells with fragmented nuclei and degradation of the F-actin cytoskeleton were often observed (Fig. 4B,B'). These alterations were also noticed following a recovery time of 3 and 6 hrs (Fig. 4C,C',D,D'). After the recovery period, actin filaments were arranged circumferentially in dense, ring-like structures or in the form of densifications or aggregations under the plasma membrane (Fig. 4D). Furthermore, F-actin became organized in dots and networks scattered throughout the cytoplasm (Fig. 4C).

DISCUSSION

Hyperthermia is a type of medical therapy in which body tissues are exposed to slightly higher temperatures to damage or make cancer cells more sensitive to other chemical or physical factors. Heat-shock destroys enzyme complexes present in the cytoplasm and mitochondria membrane but also causes alterations in enzyme system cycles, which is involved with changes in chromatin organization, regulation of gene expression and ion homeostasis [12, 15]. Our previous studies showed the effect of 41 and 44.5ºC on the morphology, ultrastructure and actin cytoskeleton of CHO AA8 cells [16, 17]. We also investigated the influence of hyperthermia (43.5 and 45ºC) on H1299 lung cancer cells [18]. In our studies, we noticed shape changes and characteristic, apoptotic features in heat-treated CHO AA8 and H1299 cells [16-18]. Furthermore, H1299 cells treated with 43.5 and 45°C heat stress showed similar changes in cell shape and membrane structure, which were probably the consequence of loss or reduction of integrins at the cell surface [18]. Hyperthermia also induces changes in actin organization, especially in switching from filamentous to monomeric state [19]. In CHO AA8 and H1299 reorganization of actin cytoskeleton was also observed [16-18]. In this paper, analyses were performed using a non-adherent human promyelocytic leukemia cell line (HL-60) and, as previously, we observed changes in the morphology and ultrastructure after heat shock treatment. It is known that mild hyperthermia promotes cell viability and proliferation [20]. In this study, we did not find any differences in viability between the control and heat-shocked cells. Following a recovery period of 3 and 6 hrs, a slight reduction in cell viability was noticed, but these data

were statistically insignificant. 3 and 6 hrs after heating, the shrunken cells with condensed and marginated chromatin were present. The cells with fragmented nuclei were also noticed. The same results were reported by Luchetti et al. (2002), who used 1 hr hyperthermia (43ºC) to induce apoptotic response of HL-60 cells, followed by a recovery time (6 hrs, 37°C) [21]. Moreover, at the ultrustructural level, the swollen mitochondria were detected. Cole et al. (1988) and Wheatley et al. (1989) observed the mitochondrial damage in cells exposed to hyperthermia [22,23]. The 43 and 45°C hyperthermia induced a decrease in pH and mitochondrial matrix density. Many researchers revealed that the effect of heat shock on the mitochondrial membrane potential is associated with a change in the cellular redox status of cells. Depolarization of mitochondrial membrane results in the reactive oxygen species (ROS) outburst [22,23]. In our previous studies with CHO AA8 or H1299 cells, apart from apoptotic cells, we observed giant cells with one enlarged nucleus or with the features of micronucleation. These morphological changes are characteristic for mitotic catastrophe [16-18]. The same results were presented by Nakahata et al. (2002), who observed tumour cells with multiple micronuclei whose number increased with the post-treatment recovery time [24]. In the present study, a few mono-nucleated giant cells were noted, but we cannot identify them as cells with the mitotic catastrophe phenotype.

Additionally, in this paper, we showed hyperthermia-induced reorganization of actin filaments. It is known that actin plays an important role not only in cell motility, membrane ruffing and formation of lamellipodia in adherent cells, but it is also involved in proliferation, differentiation, and apoptosis [13,14]. In 2003, Grzanka et al. presented a correlation between actin reorganization and apoptotic body formation during apoptosis [25]. The same scientists noticed that F-actin was seen in the nuclei of apoptotic cells [26]. Proapoptotic effect of hyperthermia has been demonstrated in various cell lines. It is known that heat shock induces changes not only in the actin cytoskeleton but also in the organization of microtubules and intermediate filaments, e.g. vimentin polymers [18]. In the present study, we observed F-actin especially at the cell periphery. We did not reveal any significant differences immediately after mild heat shock treatment (40°C/2hrs). 3 and 6 hrs after heating, we observed dense actin filaments arranged circumferentially in the ring-like structures or in the form of densifications or aggregations under the plasma membrane. We also found F-actin in form of dots and networks scattered in the cytoplasm of HL-60 cells. Our previous studies showed similar effect of cytostatic drugs (arsenic trioxide, doxorubicin, and taxol) on actin organization in HL-60 cells and confirmed that actin reorganization is associated with cell death process [25-27]. After treatment with cytostatic drugs, the actin network in the cytoplasm undergoes reorganization to form aggregates, which are necessary for the formation of apoptotic bodies. Additionally, Luchetti et al. (2002) suggested that actin could be directly involved in chromatin rearrangement occurring during apoptotic cell death [21].

In conclusion, our studies suggest that actin filament reorganization is involved in the process of apoptosis initiated by mild hyperthermia. Furthermore, we also showed that the severity of hyperthermia-induced morphological and ultrastructural changes as well as alterations in actin organization depend not only on the temperature treatment but also on the duration of post-heat shock recovery period.

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