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The influence of Trisenox on actin organization in HL-60 cells

Research Article

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Abstract: The aim of this study was to show the influence of Trisenox (arsenic trioxide, ATO) on cytoplasmic and nuclear F-actin organization in HL-60 human leukemia cell line. Changes in localization were determined with the use of fluorescence microscopy and flow cytometry. Alterations, in both cytoplasmic and nuclear actin, were observed in cells exposed to ATO. F-actin network underwent accumulation and formed aggregates, that were very often placed under the cell membrane in whole cells and at the periphery of isolated nuclei. Addition of ATO also induced apoptosis and a decrease in G2 phase cells. These results suggest the influence of actin on the formation of apoptotic bodies and also participation of this protein in apoptotic alterations within nuclei, *i.e.* chromatin reorganization.

Keywords: *F-actin • HL-60 • Trisenox (arsenic trioxide, ATO) • Apoptosis • Fluorescence • Flow cytometry*

1. Introduction

Arsenic trioxide (As_2O_3, ATO) has been used as a therapeutic agent for centuries. Fast development of pharmaceutical industry between 17th and 19th century resulted in discovery of new drugs containing ATO used to treat diseases such as skin malignant neoplasm and acute promyelocytic leukemia [1,2]. The effectiveness of ATO against acute promyelocytic leukemia (APL) was first demonstrated by Chen *et al.* in 1996 [3], who observed complete remission of APL refractory to all-trans retinoic acid under the ATO treatment. Currently, ATO has a well established role for the treatment of APL and it has been elucidated that it takes part in induction of differentiation and apoptosis in APL cells [4]. Nowadays, ATO is often used against APL in combined therapy with retinoid acid, ascorbic acid and growth factors [5-7].

The apoptotic effect of ATO has been observed in various cell types, such as acute myeloid leukemia, T-cell leukemia, lymphoma, gastric and breast cancer, glioma, prostate and ovarian carcinoma; however, the exact mechanism of its cytotoxic effect is still controversial [1,8-14]. It is known that it involves alterations of the activity of JNK kinases, and effects on NFκB transcription factor, glutathione, caspases as well as on several proapoptotic (Bax, Bak, Bid) and antiapoptotic proteins (Bcl-2, Bcl-X_L) [15-19]. While Chen *et al.* suggested that ATO induces apoptosis by degradation of the PML/RARα fusion protein and downregulation of bcl-2

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expression [3], other reports propose that its apoptotic effect is independent of PML/RARα and bcl-2, but rather depends on modulation of the glutathione redox system [1].

The cytoskeleton is an intracellular structure, consisting of three fibrillar systems in animal cells: microfilaments (actin filaments), microtubules and intermediate filaments. Actin, as the major cytoskeletal protein ubiquitous in eukaryotic cells, plays the important role in cell locomotion, membrane ruffling and formation of lamellipodia [20]. Globular actin (G-actin) is capable of forming filamentous homopolymers (F-actin). Polymerization of actin filaments mediates a large number of cellular processes, including cytokinesis, endocytosis and chemotaxis [21]. Moreover, actin cytoskeleton plays important roles in morphological changes of cell surface and in two opposing processes: differentiation and apoptosis. It was suggested that during the apoptosis there is relationship between actin and membrane blebbing, margination of nuclear chromatin and between bodies containing recognizable nuclear fragments [22-24]. In the past actin was thought to be localized only in the cytoplasm, however several studies report its presence in the nucleus [25-30]. While the function of nuclear actin is still unknown, it is suggested that it may contribute to both chromatin reorganization and apoptosis [23,31-33].

HL-60 is p53-null human acute leukemia cell line consisting predominantly of promyeloid cells, which undergo apoptosis when exposed to apoptotic factors including arsenic trioxide. The aim of this study was to investigate the rearrangement of F-actin in whole cells and isolated nuclei of the HL-60 cell line, after treatment with Trisenox (As_2O_3, a drug used in the treatment of APL), using fluorescence microscopy and flow cytometry.

2. Experimental Procedures

2.1 Cell culture

The human leukemia cell line HL-60 (ATCC CCL 240) was grown at 37º C in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 50 µg/ml gentamycin. Cell viability was assessed by the trypan blue dye exclusion method. Cell viability was assessed in 10 independent studies by the trypan blue dye exclusion method (Table 1).

Dose (µg/ml)	Mean (%)	Standard variation
0.0	95.2	1.37
0.6	90.3	3.1
1.2	81.1	3.7
2.4	48.1	4.9

Table 1. The effects of arsenic trioxide doses on cell viability.

2.2 Reagents

Trisenox (Cephalon-France), a drug containing 1 mg/ml of ATO in solution, was used. Trisenox is a water-soluble form of ATO with additional factors adjusting pH. It was added at concentrations of 0.6, 1.2 and 2.4 µg/ml of ATO in culture medium for 24 h. Control cells were grown under identical conditions, but in the absence of ATO.

2.3 Nuclear isolation

After the centrifugation, cells were suspended in cold 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 0.3 M saccharose, 3 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5% Nonidet. The mixture was homogenized on ice in Potter's homogenizer and then centrifuged (700 g, 10 min, 4˚C). Precipitate, containing fraction enriched in nuclei, was suspended in 1 ml of the solution described above and then overlaid with 10 mM Tris-HCl (pH 7.5) containing 0.3 M saccharose, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM 2-mercaptoethanol and glycerol (40%). Precipitate was dissolved in 1-2 ml of 50 mM Tris-HCl (pH 7.5) with 0.3 M saccharose, 3 mM $CaCl_2$ and 5 mM $MgCl_2$. After centrifugation (700 g, 10 min, room temperature) supernatant was poured off and the precipitate was fixed in 4% paraformaldehyde.

2.4 Fluorescence

Cells were fixed with 4% paraformaldehyde (20 min, 4°C) and then cytocentrifuged onto glass slides. 0.1% Triton X-100 was used for cells permabilization (3 min). Cells were incubated for 20 min at room temperature with phalloidin conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, Oregon, USA; methanol stock diluted 1:40 in PBS) to enable visualization of F-actin. Nuclear DNA was counterstained using 4',6-diamidino-2-phenyloindole (DAPI; Sigma-Aldrich, St. Louis, Missouri, USA). Slides were mounted in Gelvatol (Monsanto, St. Louis, USA) and an Eclipse E600 microscope (Nikon, Tokyo, Japan) was used to analyse F-actin and DNA organization.

2.5 Flow cytometry

2.5.1 Cell cycle analysis

Cells were stained with hypotonic propidium iodide solution (20 µg/ml; DNA-Prep Kit, Sigma-Aldrich, St. Louis, Missouri, USA). The analysis of 10,000 events

per sample was performed with the use of Becton Dickinson FACScan. Data analysis was carried out using FlowJo cell cycle analysis software (Tree Star).

2.5.2 Apoptosis

For the apoptosis determination, exposed phosphatidyleserine was stained with Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). Propidium iodide was used for nuclei counterstaining. Both staining processes were conducted according to manufacturer's instructions. Briefly, the cells were centrifuged (300 g, 5 min). Following the supernatant removal, 195 µl of binding buffer and 5 µl of annexin V-FITC were added. The cells were incubated in the dark (15 min, room temperature) and centrifuged (300 g, 5 min). The supernatant was removed and 190 µl of binding buffer and 10 µl of propidium iodide were added to the cell pellet. Then, the cells were incubated in the dark for 5 min. Untreated cells grown in RPMI 1640 medium supplemented with 10% fetal bovine serum served as a negative control. For the positive control, cells were exposed to 40% ethanol for 30 min. Early apoptosis was estimated in relation to the relative amount of $FITC^{+}PI^{-}$cell populations. The fluorescence of 10,000 events per sample was analyzed using a Becton Dickinson FACScan flow cytometer.

2.5.3 F-actin content

Cells were fixed with 4% paraformaldehyde (20 min, 4°C) and afterwards rinsed with PBS and permeabilized for 3 min with 0.1% Triton X-100. After washing in PBS, material was stained for 20 min at room temperature with Alexa Fluor 488-phalloidin (Molecular Probes, Eugene, Oregon, USA). Further analysis of samples was conducted with the use of Becton Dickinson FACScan flow cytometer.

2.5.4 Statistical analysis

STATISTICA 6.0 for Windows (StatSoft software, Tulsa, OK, USA) was used for the statistical analysis. All comparisons based on ANOVA Repeated Measures Designs and were presented as Duncan's test results.

3. Results

Apoptosis induced by Trisenox was related to morphological changes within cells. Alterations in the organization of actin cytoskeleton were seen in every case studied, *i.e.* from the lowest to the highest dose of arsenic trioxide, excluding the control group.

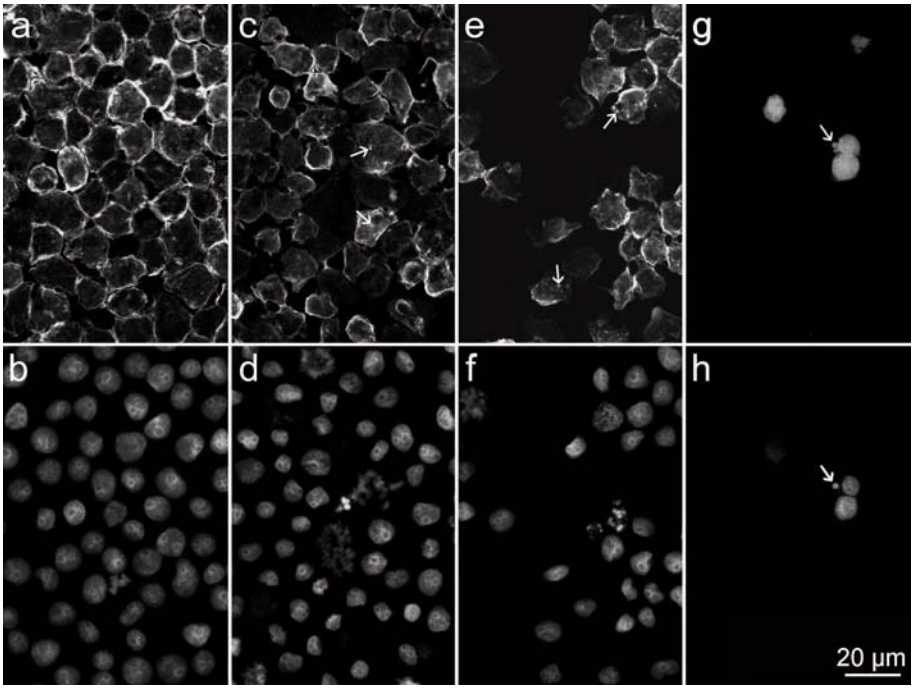

Figure 1. Fluorescence microscopy studies of actin cytoskeleton organization after ATO treatment in HL-60 cells. In control cells F-actin was localized mostly around the cell periphery. After ATO treatment aggregates of F-actin were formed and localized within the cytoplasm. Arrows indicate aggregates of F-actin and apoptotic bodies. 1ab – control; 1cd – 0.6 µg/ml; 1ef – 1.2 µg/ml; 1gh – 2.4 µg/ml; (a, c, e, g) – F-actin labeling; (b, d, f, h) – DAPI labeling.

In HL-60 control cells we could see F-actin forming a structure localized around the cell periphery. There were also little fluorescence signals, in the form of network and small aggregates, in the cytoplasm (Figure 1a). Even the lowest dose of Trisenox (0.6 μg/ml) triggered effects on the actin cytoskeleton. In most cases F-actin shifted from the form of network to small aggregates localized within the cytoplasm. However, cells with well-preserved F-actin networks and those with both forms (cytoplasmic aggregates and the network) were also observed (Figure 1c). At medium dose (1.2 μg/ml) of Trisenox, alterations in the arrangement of actin were more visible, as we observed less networks but more aggregates of actin, as compared to the lowest dose (Figure 1e). Only few cell nuclei showed apoptotic features, which was shown by DAPI staining (Figure 1f). Finally, Trisenox in concentration of 2.4 μg/ml induced apoptosis in most treated cells, which caused the formation of actin aggregates within the cell or in emerging apoptotic bodies (Figure 1g). With increasing ATO dose, the number of cells decreased rapidly and their shape became more irregular.

Moreover, fluorescence microscopy studies showed changes in F-actin localization in isolated nuclei. In control cells, F-actin was localized at the periphery of the nucleus as well as in its whole area, where it formed network (Figure 2a). Trisenox in concentration of 0.6 μg/ml caused actin accumulation in the form of granular structures, in the absence of network (Figure 2c). The cell nucleus underwent deformation and degradation (Figure 2d). F-actin was visible as granulated structures, which overlapped with DAPI staining (Figure 2c,d). At the 1.2 μg/ml Trisenox concentration there was no network and actin was also localized in the form of granular structures (Figure 2e). Nuclei were not isolated for the 2.4 μg/ml Trisenox concentration, as there was a large percentage of apoptotic cells in the stage of nuclei disintegration at this dose.

Trisenox not only changed the localization of F-actin within the cell, but also its amount. Flow cytometry analysis demonstrated a considerable increase in fluorescence intensity after the application of Trisenox doses, as compared to the control group. The highest fluorescence intensity was observed at the lowest Trisenox dose and decreased together with a dose. However, the fluorescence intensity of cells after treatment with the highest Trisenox dose (2.4 μg/ml) was still higher than that of the control cells (Figure 3, 4).

Fluorescence intensity of actin on the level of the cell nucleus after treatment with 0.6 μg/ml ATO was lower than that observed in the control cells. At the Trisenox dose of 1.2 μg/ml, fluorescence intensity was higher in comparison to the lower ATO dose. Concurrently,

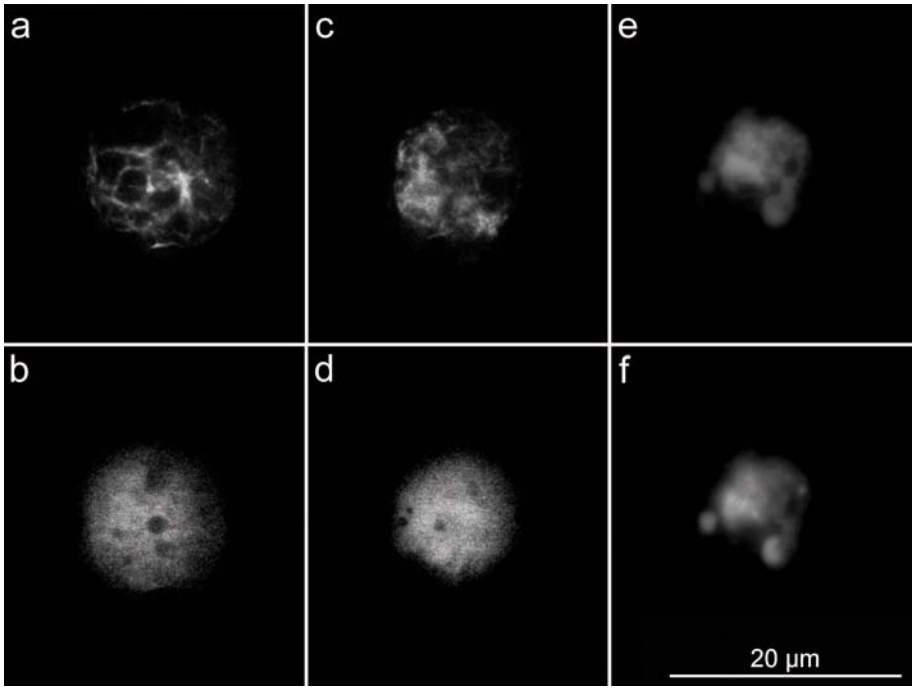

Figure 2. Fluorescence microscopy studies of F-actin localization changes in isolated nuclei after ATO treatment in HL-60 cells. In isolated nuclei from control cells F-actin formed network localized in the nucleus periphery. After ATO treatment F-actin formed granular structures. 2ab – control; 2cd – 0.6 μg/ml; 2ef – 1.2 μg/ml; (a, c, e) – F-actin labeling; (b, d, f) – DAPI labeling.

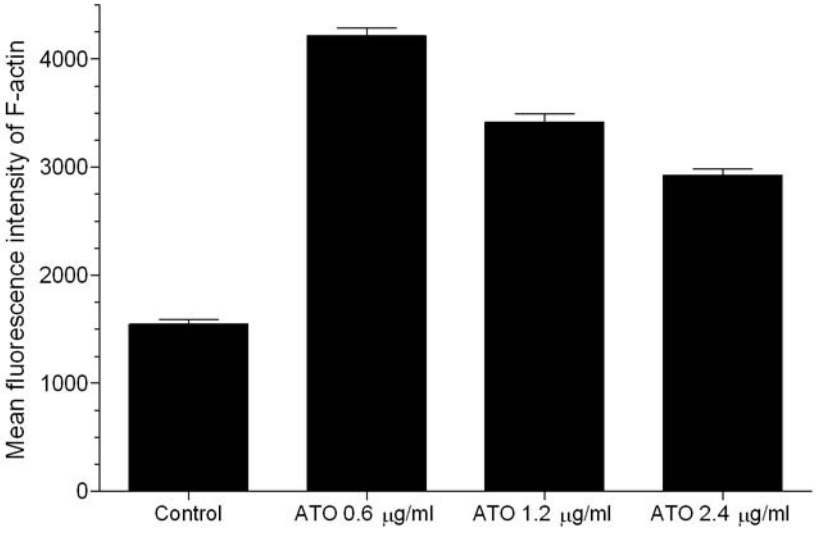

Figure 3. Bar charts illustrating the mean fluorescence intensity of F-actin in HL-60 cells after ATO treatment. Cells were treated with ATO for 24 h at concentrations: 0.6, 1.2 and 2.4 μg/ml. Analysis of F-actin in HL-60 showed changes of fluorescence intensity. The highest fluorescence intensity was observed at the lowest ATO concentration and decreased together with the ATO dose. The fluorescence intensity at the highest ATO dose was higher than that of the control cells.

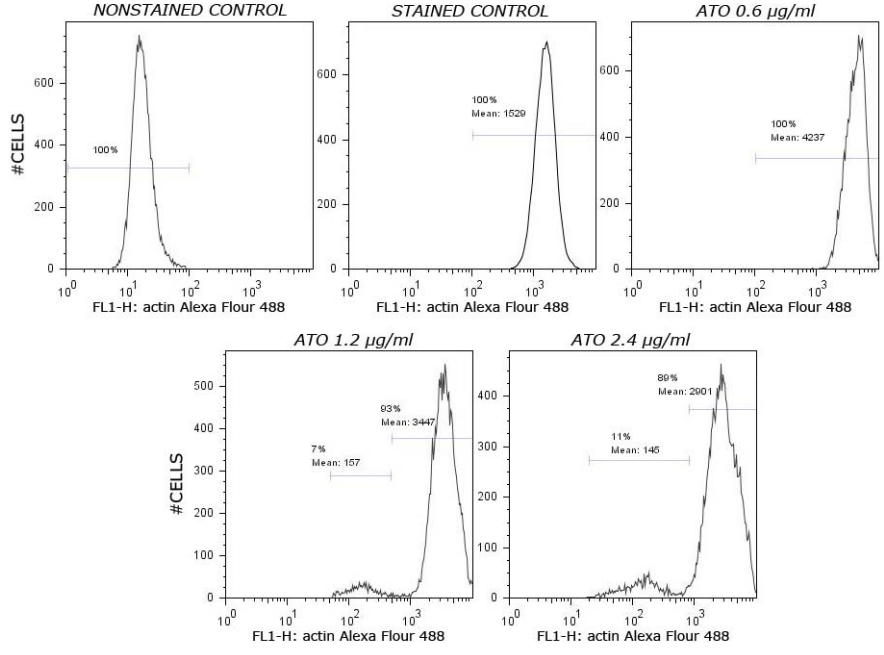

Figure 4. Representative flow cytometric analysis of F-actin content in HL-60 cells after ATO treatment. Cells were treated with ATO for 24 h at concentrations: 0.6, 1.2 and 2.4 μg/ml. Analysis of F-actin in HL-60 showed changes of fluorescence intensity. The highest fluorescence intensity was observed at the lowest ATO concentration and decreased together with the ATO dose. The fluorescence intensity at the highest ATO dose was higher than that of the control cells.

a part of very highly fluorescent cell nuclei increased dose-dependently (Figure 5). There were statistically significant differences in fluorescence intensity for both, cell nuclei and whole cells (Duncan's test, $P<0.05$). One of the methods of apoptosis detection is the analysis concerning translocation of the membrane phospholipid phosphatidylserine, using fluorescently labeled Annexin V. In the control, 7% of cells were apoptotic. For the lowest (0.6 µg/ml) and medium (1.2 µg/ml) doses of Trisenox, the content of apoptotic cells increased to 11 and 17%, respectively. The highest dose of Trisenox (2.4 µg/ml) induced apoptosis in 50% of the studied population (Figure 6). Cell cycle analysis, demonstrated a decrease in the percentage of G1 cells in aid of the S phase, in relation to control cells after treatment with Trisenox (Figure 7). This effect was directly proportional to the dose of ATO.

4. Discussion

The work presented here is based on the studies of F-actin organization in HL-60 cell line, which was treated with proapoptotic factor - Trisenox (arsenic trioxide, As_2O_3, ATO). Proapoptotic effect of arsenic trioxide has been demonstrated with various cell lines [8-10,15,34,35] and it is known that ATO induces changes in actin cytoskeleton. The main objective of this work was to study the effect of arsenic trioxide on a cytoskeleton protein - F-actin. Our results showed changes in F-actin arrangement after treatment with Trisenox, both in the cytoplasm and in the nucleus. In the control, F-actin was localized on the edges of the cell and formed a network within it, similarly to the epithelial cells studied by Zhang [36]. Previously, Shen *et al.* demonstrated that in response to ATO actin network in the cytoplasm of HL-60 and SHEE cell lines undergoes reorganization to form aggregates [37]. At the highest Trisenox dose, accumulation of F-actin on the cell peripheries is probably connected to the formation of apoptotic bodies. Our previous studies confirmed that actin polymerization is necessary during the formation of apoptotic indents in cell membrane and shaping of apoptotic structures is related to a local reorganization of this protein [22,38]. After the treatment with doxorubicin and taxol in HL-60 and K-562 human leukemia and CHOAA8 cell lines, rich F-actin labeling was demonstrated in places where apoptotic bodies occurred [22,38].

The quantity of F-actin was also altered by Trisenox. Zhang and Veselska showed a decrease in F-actin level as a response to higher doses of ATO [36,39]. In HL-60 cells, an increased amount of F-actin was observed at the

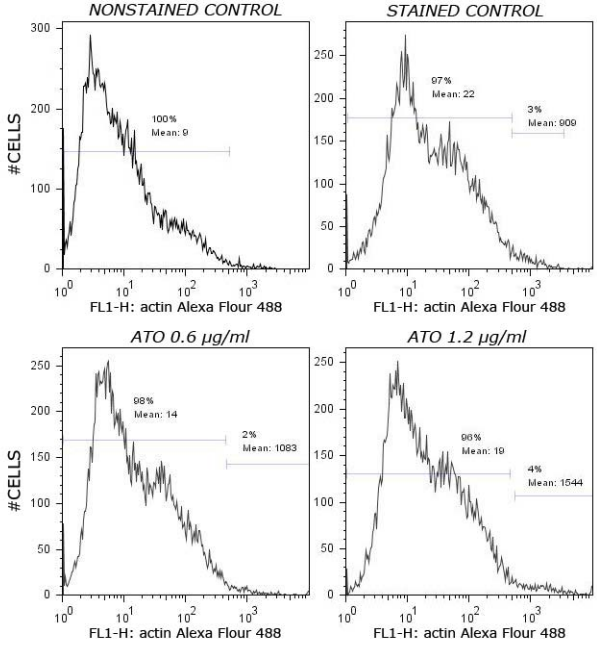

Figure 5. Flow cytometric analysis of F-actin content in cell nuclei in HL-60 cells after ATO treatment. Cells were treated with ATO for 24 h at concentrations: 0.6, 1.2 µg/ml. Isolated cell nuclei were stained with phalloidin conjugated to Alexa 488. Fluorescence intensity analysis of F-actin in cell nuclei showed decrease at 0.6 µg/ml dose with reference to control and increase at dose 1.2 µg/ml in comparison to the lowest ATO dose.

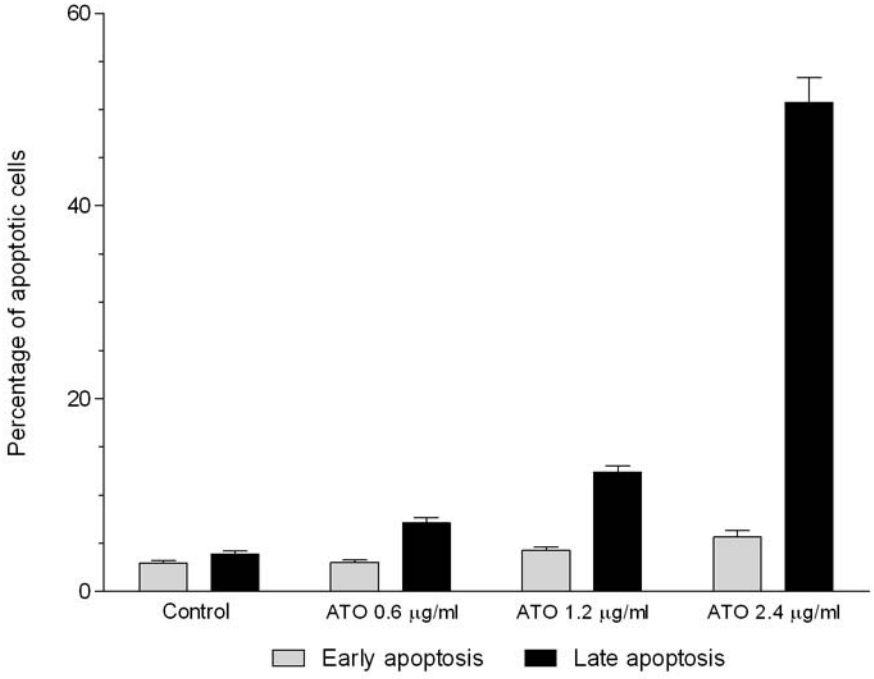

Figure 6. The percentages of apoptotic cells in HL-60 cell line after treatment with ATO. Cells were treated with ATO for 24 h at concentrations: 0.6, 1.2 and 2.4 μg/ml. Together with increasing ATO dose, an increase of apoptotic cells content was observed.

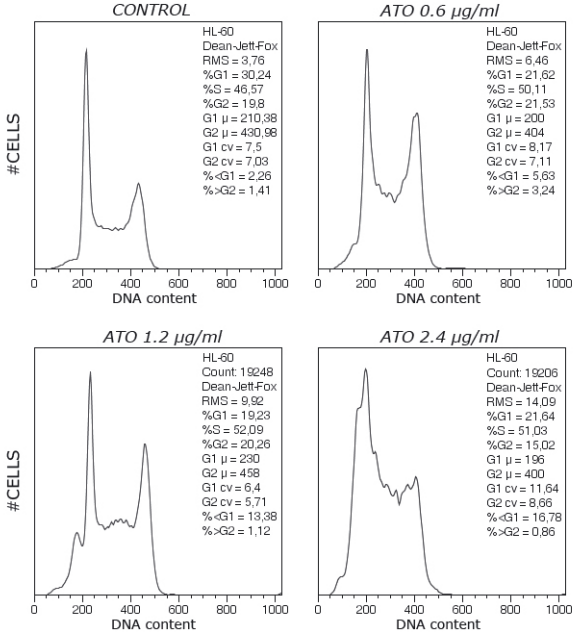

Figure 7. Flow cytometric analysis of HL-60 cells cell cycle after ATO treatment. Cells were treated with ATO for 24 h at concentrations: 0.6, 1.2 and 2.4 μg/ml. Cell cycle analysis showed the decrease in percentage of G1 and G2/M phase cells and increase of the S phase with regard to the control.

lowest doses. Other researchers suggest gradual F-actin depolymerization, together with an increase of the ATO dose [37]. Our experiments with HL-60 cells revealed similar effects. However, gradual depolymerization in relation to the minimal Trisenox dose occurred, but not in the control cells. The increased F-actin polymerization at the lowest dose of ATO might be due to rapid changes that take place in the cell. While some researchers suggest that actin cytoskeleton participates only in the differentiation process [40], our results demonstrate changes in localization and fluorescence intensity of F-actin, which imply actin involvement in the process of apoptosis.

Our previous studies indicate that F-actin is present in the cell nuclei in the proximity of chromatin [38,41], suggesting that it may be involved in chromatin reorganization during the formation of morphological features typical of apoptotic cells. Application of the proapoptotic factor, Trisenox, caused chromatin condensation and formation of characteristic F-actin aggregates. Alterations of the F-actin fluorescence intensity within nucleus are most likely connected with intensive changes that occurred in the nucleus area and with F-actin participation in chromatin rearrangement. It was reported that nuclear actin exists in several unusual conformations which are different from those typical of actin filaments in the cytoplasm [42-45]. Furthermore, FRAP analysis has shown that ~20% of the total nuclear actin pool has polymeric actin features [46]. Zhao *et al.* pointed the direct participation of F-actin in chromatin reorganization, by its presence in BAF complex (multilateral complex bounding actin) where actin could act as a nucleotides shifting factor [31]. Starr and Han suggested that actin connects with the nucleus through ANC-1 (domain, binding actin with the nucleus) and UNC-84 protein, present in the nuclear membrane [47]. Results suggest that F-actin is connected with nuclear membrane proteins, as it is present in nucleus peripheries, besides its internal localization. There are also reports on nuclear actin being associated with nucleoplasmic filaments in nuclear pore complex and participating in transportation, not only retroviral RNA, but also protein kinase inhibitor (PKI) [48]. Our fluorescence microscopy results suggest that the surface localization of F-actin may be involved with nuclear pore in control cells. Apoptotic reorganization and changes of F-actin fluorescence intensity could be engaged in chromatin reorganization and nuclear fragmentation during apoptosis. It was also suggested that F-actin plays important roles in relocation of RNA transcript and in establishing chromosomal area, which limits the mobility of chromatin fragments [45,49]. Local changes in distribution and quantity of nuclear F-actin after treatment with ATO may be related to alterations to such processes.

In addition to the influence of arsenic trioxide on apoptosis induction and subcellular distribution of actin, its effect on cell cycle phases was also studied. Cell cycle analysis after Trisenox treatment demonstrated a decrease in the percentage of G1 phase cells and an increase of the S phase, in proportion to control. Increased numbers of cells in G2/M phases, induced by ATO, was also observed by Liu *et al*. in myeloma cells and Park *et al.* in U937 cell line [8,50]. However, studies in other cell lines indicate the inhibition of cell cycle in G1 phase [36]. A blockade in particular cycle phase has been shown to be dependent on the duration and the dose of cytostatic and experimental cell line used [9,36,50]. Studies conducted on seven myeloma cell lines indicated that it is also dependent on the genetic background *i.e*. whether the cell line is of wild type or it has gene mutation such as p35 or p21 [8,51]. Studies on HL-60 line carried out by Zhang *et al.* indicate a minimal increase in the number of cells in G2/M phases, after three days and in G0/G1 phases after five days of ATO treatment [36].

In conclusion, this study shows that arsenic trioxide influences the actin cytoskeleton and the reorganization of nuclear actin. Arsenic trioxide causes also the decrease in G2/M phases of the cell cycle in HL-60 cells and induces apoptosis.

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