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THE EFFECTS OF CAFFEINE ON CYTOSKELETON IN CHO AA8 CELL LINE

WPŁYW KOFEINY NA CYTOSZKIELET LINII KOMÓRKOWEJ CHO AA8

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S u m m a r y

The caffeine is one of the most widely consumed alkaloids, which is found in energy drinks, coffee, tea, cocoa and chocolate. This neuroactive substance is a component of the daily diet of millions people in the world. For many years, the attention of researches focused on the effects of caffeine on the living organism, especially in the context of physiological processes. Today, the mechanism of the action of caffeine is the object of the studies.

The cytoskeletal proteins play an important role in cell death. In our work, we analyzed changes in vimentin, F-actin and β-tubulin cytoskeleton. Furthermore, alternations in morphology and ultrastructure were also evaluated. We used fluorescence and light microscopy and transmission electron microscopy as well.

Our data showed that the treatment with caffeine resulted in decreased in the survival of CHO cells. Moreover, we noticed two types of cell morphology: giant, multinucleated cells and shrunken cells with chromatin condensation. This investigation suggested that caffeine may induce apoptosis and mitotic catastrophe cell death.

S t r e s z c z e n i e

Kofeina to jeden z najczęściej spożywanych alkaloidów, spotykany między innymi w napojach energetyzujących, kawie, herbacie czy czekoladzie, przez co stanowi ona składnik codziennej diety milionów ludzi. Przez wiele lat uwaga naukowców skupiała się na działaniu kofeiny na organizmy żywe, w tym procesy fizjologiczne. Od niedawna, przedmiotem badań jest mechanizm działania kofeiny.

Ze względu na zaangażowanie białek cytoszkieletu w procesy śmierci komórkowej, za zasadne uznano ocenę indukowanych przez alkaloid zmian w obrębie wimentyny, F-aktyny oraz β-tubuliny. Ponadto analizowano morfologię i ultrastrukturę komórek badanej linii. Podczas realizacji tematu wykorzystano metodykę z zakresu mikroskopii świetlnej, klasycznej mikroskopii fluorescencyjnej, a także transmisyjnej mikroskopii elektronowej.

Uzyskane wyniki wskazują, iż kofeina wpływa istotnie statystycznie na przeżycie komórek linii CHO AA8. Obserwowane dwa typy morfologii komórek – obkurczonych, ze skondensowaną chromatyną, a także olbrzymich z widocznymi mikrojądrami, sugeruje, iż alkaloid może indukować zarówno apoptozę, jak i katastrofę mitotyczną.

Key words: caffeine, vimentin, F-actin, tubulin, apoptosis, mitotic catastrophe
Słowa kluczowe: kofeina, CHO AA8, vimentyna, F-aktyna, tubulina, apoptoza, katastrofa mitotyczna

INTRODUCTION

Caffeine is neuroactive compound of tea leafs and coffee beans. Caffeine is a natural alkaloid, derivative of xanthines, which has been characterized by three methyl groups: 1,3,7-trimethylxantine [1].Marta Hałas et al. Caffeine has been reported to affect regulatory machinery of cell cycle via several molecular targets. It has been also shown that caffeine could inhibit cell growth, and induce G1 phase arrest while lower concentrations cause cell arrest in G0 phase [2,13]. On the other hand caffeine at concentration up to 4mM abrogate G2/M checkpoint, whereas higher doses lead up to direct apoptosis [3,4].

Inside cell, the cytoskeleton forms intricate, dynamic structure, which functions are crucial for cell viability and proliferation. It consists of 3 types of filaments: microfilaments, intermediate filaments, and microtubules. In general, they are responsible for cell properties such like shape and plasticity, organelles movement and signals transduction. Actin occurs in two forms: unpolimerized globular G-actin (Globular Actin), and polimerized F-actin (Filamentous Actin). Its polymerization is controlled by many regulatory proteins such as Rho GTPases and actin-associated proteins (MAPs) [5]. Intermediate filaments (IFs) constitute a large protein family, which help in maintenance of cell shape and anchorage of some cell organelles. Six types of IFs can be distinguished: types I and II – acidic and basic keratins, type III - vimentin and desmin, type IV – neurofilaments, type V – lamins and type VI-nestin [6]. Lamins are proteins which play a crucial role in maintenance of nucleus structural properties. There are two types of lamins in eukaryotic cells: B-type lamins (which fall into two subtypes: lamin B1 and lamin B2) and lamin A/ C. Lamins are involved in such cellular processes like regulation of nuclear shape and mechanical stability, regulation of chromatin positioning and gene expression, mitosis and maintenance of genome stability [7,8]. Microtubules are polymers consisting of α- and β-tubulin units. Microtubules are responsible for maintenance of cell polarity, intracellular transport and cell division [9]. The aim of this study was to determine the influence of caffeine on alterations in the main cytoskeletal proteins and the type of induced cell death in Chinese Hamster Ovary cell line (CHO AA8).

MATERIALS AND METHODS

Cell culture

The Chinese Hamster Ovary cell line (CHO AA8) was purchased from American Type Culture Collection (ATCC). Cells were grown in MEM (Modified Eagle's Medium) and supplemented with gentamycin and 10% fetal bovine serum (FBS) at 37 ^{0}C with 5% CO_2. After reaching the 80% confluence, cells were removed from cell culture dishes with trypsin–EDTA solution and subcultured on 12- or 6-well plates for further investigations. The CHO AA8 were treated with caffeine at concentration 10 and 20 mM for 24h. The control cells were grown under the same condition without alkaloid.

Annexin V-FITC/PI double staining

Cell viability and cell death were measured using Tali® Apoptosis Kit (Invitrogen) according to manufacturer's instructions. The CHO AA8 cells treatment with caffeine were stained with Annexin V FITC and Propidium Iodide. The cells were resuspended in 100 µl ABB (annexin binding buffer, Invitrogen). After the addition of Annexin-V (20 minutes at RT, in the dark) and PI (5 min, RT in the dark), the cell suspension was gently vortexed and analyzed by Tali® Image-Based Cytometer.

Immunofluorescence of β-tubulin, vimentin, F-actin

Immunostaining of cytoskeletal filaments was performed on the basis of the protocol previously described by Safiejko-Mroczka and Bell (1998). Cells were seeded on coverslips in 12-well plates and then treated with caffeine (at concentration 10 and 20 mM) for 24 hours, excluding control cells. In order to β-tubulin staining, cells were prefixed with 1 mM 3,3'-dithiodipropionic acid di(N-hydroxysuccinimide ester) (DTSP; Sigma–Aldrich; Buchs, Switzerland), which is a bifunctional protein crosslinking reagent, diluted in Hanks' Balanced Salt solution (HBSS; 1:50; Sigma–Aldrich; St. Louis, MO) for 10 min at RT. Next, cells were pre-extracted with Tsb [0.5% Triton X-100 (Serva; Heidelberg, Germany) in microtubule stabilizing buffer (MTSB: 1 mM EGTA, 10 mM PIPES, 4% poly(ethylene glycol); Sigma–Aldrich; St. Louis, MO)] containing DTSP (dilution 1:50) for 10 min at RT. After incubation with pure Tsb (5 min, RT) and fixation with 4% paraformaldehyde (Serva; Heidelberg, Germany) in MTSB (15 min, RT), cells were treated with 1% BSA–PBS [bovine serum

albumin (Sigma–Aldrich; St. Louis, MO) in Tris-buffered saline; pH 7.6] 2 x 5 min at RT. β-tubulin was labeled using a mouse monoclonal antibody specific for β-tubulin (diluted 1:65 in 1% BSA–TBS; Sigma–Aldrich) for 60 min at RT in absence of light. For examination of vimentin cells were fixed by 4% paraformaldehyde (Serva). The vimentin staining was performed using a mouse monoclonal antibody specific for a vimentin (diluted 1:200 in 1% BSA; Sigma–Aldrich) for 60 min at RT in the dark. Afterwards coverslips were washed three times for 5 min with PBS and incubated with TRITC-labeled goat anti-mouse secondary antibody (Sigma–Aldrich) diluted in BSA (1:85) for 60 min at 37 C. F-actin was labeled using Alexa Fluor 488 coniugated with phalloidin (1:40; Invitrogen, Molecular Probes) for 20 min at RT. After rinsing with PBS (3x5 min), cell nuclei were stained using 4',6-diamidino-2-phenyindole (DAPI; 10 min; Sigma–Aldrich;). Finally, cells were washed three times with PBS and mounted in Aqua-Poly/Mount (Polysciences). The organization of cytoskeletal filaments was analyzed using a Nikon Eclipse E800 fluorescence microscope (Nikon; Tokyo, Japan) and NIS-Elements 4.0 software (Nikon).

Transmission electron microscopy

The CHO AA8 cells were fixed with 3.6% glutaraldehyde, washed in cacodylic buffer (0.1M pH = 7.4), postfixed with 1% osmium tetroxide, dehydrated with an ascending series of alcohols and acetones, and finally embedded in Epon 812. Selected parts of material were cut into ultra-thin sections by using a Reichert OmU3 ultramicrotome and then counterstained with uranyl acetate. For the ultrastructural examined, material was analyzed using JEM 100 CX electron microscope (JEOL).

Statistical analysis

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

RESULTS

Cell death and viability of CHO AA8 after caffeine treatment

The Annexin V/ Propidium Iodide test revealed that CHO AA8 cells exhibited susceptibility to the cytotoxic effect of caffeine treatment. As shown in Figure 1A, the mean percentage of viable cells was 95.35% in control cells, 89.94% after 10 mM and 82% following 20 mM treatment with caffeine. The data showed statistical differences in the population of apoptotic cells after caffeine at concentration 20 mM compared to untreated cells (CTRL – 1.49%, 20mM – 11.2%; Fig. 1B).

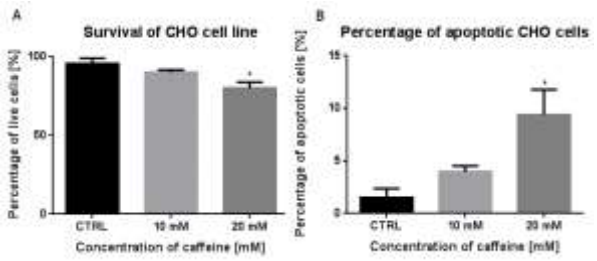


Fig. 1. *The influence of caffeine on: A - the average percentage of viability CHO AA8 cells; B- the percentage of apoptotic cell*

Rys. 1. *Wpływ kofeiny na: A- przeżycie komórek linii CHO AA8; B- procent komórek apoptotycznych*

The effects of caffeine on the morphology and ultrastructure of CHO AA8

The light microscope was used to examine alternations in the morphology of CHO AA8 cell line. The cells untreated with caffeine had shape typical for fibroblast (Fig. 2A). Following the treatment with 10 mM caffeine, the cells were characterized by large size, and multinucleation (Fig.2B). These features are characteristic of mitotic catastrophe (mitotic death). The treatment with caffeine at concentration of 20 mM resulted in the presence of giant cells with large, fragmented nucleus (Fig.3C). Furthermore, in both dose of alkaloid the shrunken cell with the condensation of chromatin were also observed (data not shown).

The electron microscopy studies revealed the ultrastructural changes in CHO AA8 cell line. In the control group, the cells were characterized by regular shape and oval nuclei (Fig. 3A). The incubation with caffeine at concentration of 10 mM resulted in shrunken nuclei and a lot of vacuoles in the cytoplasm. The swollen mitochondria and cells with micronucleus were also observed (Fig. 3B,C). The highest doses of alkaloid caused abnormal nuclear shape and cell membrane rupture (Fig. 3D). The presence of apoptotic blebs was also noticed (Fig. 3E).

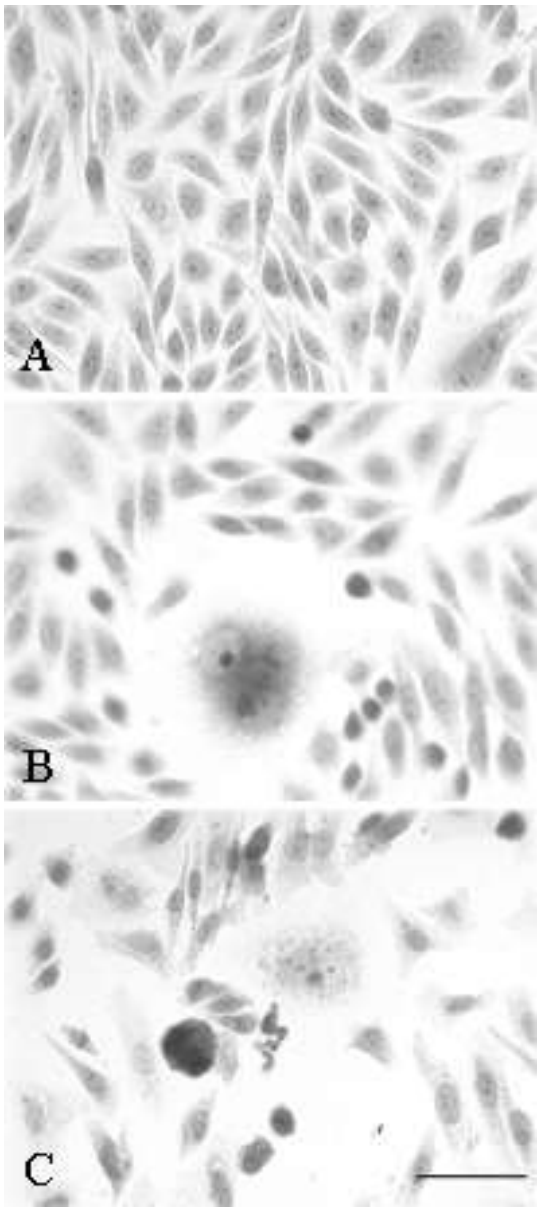
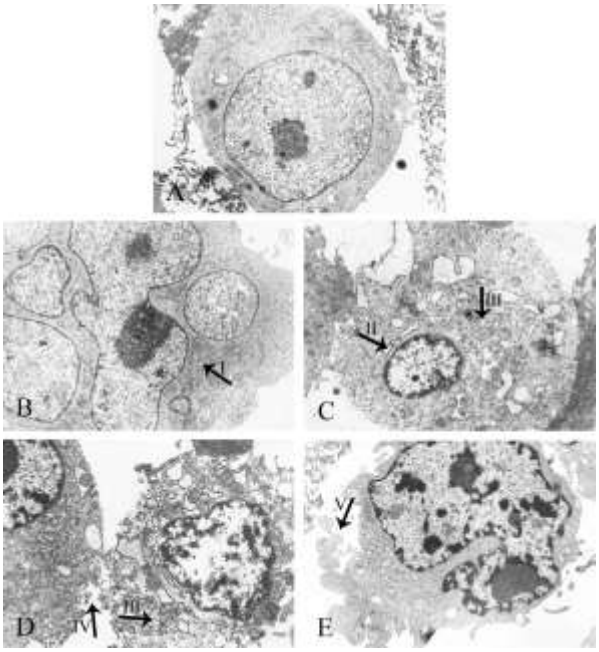


Fig. 2. *The effect of caffeine on the morphology in the CHO AA8 cells. A- control, B- CHO AA8 cells incubated with caffeine at a dose of 10 mM, C- CHO AA8 cells incubated with caffeine at a dose of 20 mM. The apoptotic cell and cell with mitotic catastrophe phenotype were observed. Bar - 50 μm*

Rys. 2. *Wpływ kofeiny na morfologię komórek CHO AA8. A – komórki kontrolne; B – Komórki CHO AA8 traktowane kofeiną w stężeniu 10mM; C- 20 mM; Widoczne komórki apoptotyczne oraz komórki o fenotypie katastrofy mitotycznej. Bar - 50 μm*



Rys. 3. *The effect of caffeine on the ultrastructure in the CHO AA8 cells. A- control, magnification x5000; B,C- CHO AA8 cells incubated with caffeine at a dose of 10mM, magnification x5000; D,E - CHO AA8 cells incubated with caffeine at a dose of 20 mM, magnification x5000; The micronucleated cells (I), the swollen mitochondria (III) and shrunken nucleus were observed (II). We noticed the cell membrane reptures (IV) and apoptotic blebs (V)*

Rys. 3. *Wpływ kofeiny na ultrastrukturę komórek CHO AA8. A- komórki kontrolne, powiększenie x5000; B,C – komórki CHO AA8 traktowane kofeiną w stężeniu 10 mM, powiększenie x5000. D, E- 20 mM kofeiny, powiększenie x5000; Widoczne komórki z mikrojądrami (I), nabrzmiałymi mitochondriami (III) oraz obkurczonym jądrem komórkowym. Obserwujemy komórki z przerwaną błoną komórkową (IV) oraz pączkami apoptotycznymi (V)*

The changes in the main cytoskeletal proteins in the CHO AA8 (F-actin, β-tubulin and vimentin)

The classical fluorescence staining was used to evaluate changes in the main cytoskeletal proteins. For staining of F-actin, the Phalloidin-Alexa Fluor 488 conjugate was used. The control of CHO AA8 cells was characterized by arrangement of F-actin on the edge of the cells. As showed in Figure 4D (after 10 mM caffeine), the shrunken cells with depolymerization of F-actin were observed. Furthermore, a lot of stress fibers and the nuclei with condensation of chromatin were also seen (Fig. 4D). After the exposure to caffeine, the CHO AA8 cells with mitotic catastrophe phenotype were noticed. In these cells, F-actin was degraded and cell nuclei were

strongly fragmented (after 20 mM caffeine; Fig. 4G'). In the other hand, as shown in Fig. 4G, the giant cells with a lot of stress fibers and large nucleus were seen.

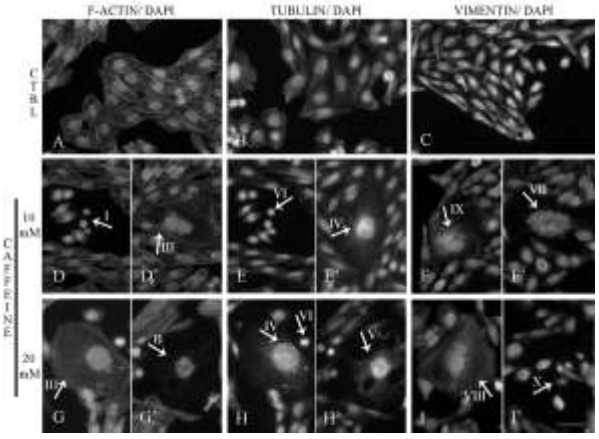


Fig. 4. *The effect of caffeine on the F-actin, vimentin, β-tubulin organization in the CHO AA8 cells. The F-actin: A – control cells; D,D' – 10 mM of caffeine; G,G' – 20 mM of caffeine. The depolymeryzation of F-actin (I) and small aggregates (II) were noticed. Furthermore, giant cell with stress fibers were also seen (III). The β-tubulin: B- control; E,E' – cells incubated with 10 mM caffeine; H,H'- CHO AA8 treated with 20 nM caffeine. The microtubules in the form of crescent-like around the nucleus were noticed (IV). Moreover, small aggregates and short polymers of β-tubulin were also seen (V). We noticed the depolymeryzation of microtubules (VI). The vimentin: C – CHO AA8 control cell; F,F' – caffeine-treated CHO AA8 at concentration 10 mM; I,I' – 20 mM caffeine. We observed the reorganization (VII) and diffuse of vimentin (VIII) and cell with accumulation of vimentin around the nucleus (IX). The depolymeryzation of vimentin were also noticed (X). Bar - 50 μm*

Rys. 4. *Wpływ kofeiny na organizację F-aktyny, wimentyny oraz β-tubuliny. F-aktyna: A – kontrola CHO AA8; D,D' – komórki CHO AA8 traktowane 10 mM kofeiny; G,G' – 20 mM kofeiny. Widoczna depoliemryzacja F-aktyny (I) oraz małe agregaty (II), a także olbrzymie komórki z włóknami stresowymi (III). β-tubulina: B – kontrola; E,E' – dawka 10 mM kofeiny; H,H' – 20 mM kofeiny. Widoczne mikrotubule w postaci półksiężyca, zlokalizowanego wokół jądra komórkowego (IV), a także małych agregatów i krótkich polimerów (V). Depolimeryzacja mikrotubul (VI). Wimentyna: C-kontrola CHO AA8; F,F'- stężenie kofeiny 10 mM; I,I' – 20 mM kofeiny. Widoczna reorganizacja (VII) i rozproszenie (VIII) wimentyny, a także akumulacja w okolicy jądra komórkowego (IX). Depolimeryzacja wimentyny (X). Bar - 50 μm*

In order to β-tubulin staining, the mouse monoclonal antibodies specific for β-tubulin were used. Our data showed that the treatment of CHO AA8 with caffeine resulted in the appearance of shrunken, apoptotic cells. The depolymerization of F-actin co-existed with depolymerization of microtubules (10 mM caffeine - Fig. 4E and 20 mM – Fig.4H). Although, in giant cells with large size of nucleus, tubulin in the form of crescent-like around the nucleus was seen. Moreover, we observed above mentioned features at concentrations 10 and 20 mM caffeine (Fig. 4G',H). Among the cells exhibiting mitotic catastrophe-like phenotype, the small aggregates and short polymers of β-tubulin in the cytoplasm were noticed (20 mM caffeine; Fig. 4H').

The structures of vimentin were labeled by a mouse monoclonal antibody specific for vimentin. The treatment with caffeine revealed distorted vimentin arrangement. In low doses of caffeine, we noted the presence of giant cells with the accumulation of vimentin around the nucleus (Fig. 4F) or their strong depolimerization (Fig. 4F'). After the incubation with caffeine at concentration of 20 mM the shrunken cells were characterized by the reorganization of vimentin were seen (Fig. 4I'). However, we also observed multinucleated cells with diffuse of vimentin cytoskeleton (Fig. 4I).

DISCUSSION

The cytostatic effects of caffeine have been investigated by many authors since this alkaloid is not only a very popular component of our daily diet but also a potential anticancer agent. In many experimental studies the researchers have evaluated the preventive and therapeutic effects of caffeine against various cancer cells and they have revealed that the mechanism of caffeine action may be associated with the induction of apoptosis and/or mitotic catastrophe [10]. Qi et al. presented that caffeine induced apoptosis in human lung adenocarcinoma cells [13]. Additionally, the apoptotic effect of caffeine has been also observed in the human neuroblastoma SK-N-MC cells and human pancreatic adenocarcinoma cells [12]. Furthermore, Dai et al. showed that caffeine treatment elevated apoptosis level in the human leukemia HL-60 and U937 cells via mitochondrial damage [11]. Our previous study on the human non-small lung cancer H1299 cell line has revealed that caffeine decreased

cell viability and induced apoptotic cell death in a dose-dependent manner.

In our studies, we have observed two types of cell morphology: i) the shrunken morphology typical for apoptotic cells and ii) the enlarged multinucleated morphology characteristic of mitotic death (mitotic catastrophe). The obtained data have shown that alterations in cell morphology corresponded with cytoskeletal proteins reorganization. Furthermore, the electron microscopy studies have revealed that caffeine treatment resulted in cell membrane rupture, swollen mitochondria and cytoplasmic vacuolization. Moreover, we have observed plasma membrane blebbing, which is characteristic for apoptosis. We have also demonstrated the giant mono- or multinucleated cells, which are considered to be characteristic features of mitotic catastrophe. In many papers, the authors have used the term 'mitotic catastrophe' for defining a type of cell death that occurs during mitosis as a result of DNA damage and deficient cell cycle checkpoints [14,15,16,17]. Portugal et al. presented that mitotic catastrophe is a characteristic strategy of death in cells with the lack of p53 gene expression [17]. It is known that CHO AA8 cells are defective for p53; therefore, in addition to apoptotic figures, we have also observed the structure characteristic for mitotic catastrophe. Similar results were presented by Grzanka et al. and Pawlik et al. on the H1299 cell line, which has a homozygous partial deletion of the p53 gene [18,20]. Additionally, our previous study on H1299 cells has revealed that caffeine induced not only apoptotic cell death but also mitotic catastrophe.

The second aim of our study was to determine the influence of caffeine on the F-actin, tubulin and vimentin in CHO AA8 cells. It is known that cytoskeleton plays a key role in the different cellular processes. The cytoskeleton provides mechanical support for maintaining cell shape and it is essential for such processes as intracellular transport, cell motility, cell division and apoptosis as well [21-24]. In the present study, we have observed the rearrangement of F-actin, tubulin and vimentin, which was connected with different physiological states of the cell. Our study has shown that the treatment of CHO AA8 cells with caffeine caused depolimeryzation of actin filaments in shrunken cells. In our previous study, we have also observed the degradation of actin cytoskeleton in apoptotic cells after the treatment of cancer cells with cytostatic drugs. However, in a few apoptotic cells, F-actin accumulated near the plasma membrane, what was probably related to its participation in the formation of apoptotic blebs [27]. Apoptotic bodies are the structure characteristic for apoptosis that contain cellular organelles and chromatin. Many scientists have shown that actin participates in the formation of apoptotic structures. Keller et al. as well as Cotter et al. revealed that the polymerization of actin filaments is essential during apoptotic bodies' formation [25,26]. F-actin labeling in the places of apoptotic blebs formation has been also observed after the treatment of CHO AA8, HL-60 and K-562 cells with taxol or doxorubicin [27,28].

Our fluorescence microscopy observations have revealed that in the cells with mitotic catastrophe phenotype, F-actin was degraded (small aggregates) and cell nuclei were strongly fragmented. On the other hand, giant cells with a lot of stress fibers and large nuclei were also seen. These results are similar to our previous studies with caffeine-treated H1299 cells, in which we concluded that the reorganization of F-actin is necessary not only in apoptotic process but also in other types of cell death. The present studies have also demonstrated that in the apoptotic cells the depolymerization of F-actin co-existed with depolymerization of microtubule. In turn, in the giant cells with large nucleus, tubulin in the form of crescent-like structures around the nucleus was seen. Furthermore, among the cells with mitotic catastrophe phenotype, the small aggregates and the short polymers of β-tubulin in the cytoplasm were also noticed. Similar observations were presented by Pawlik et al., who revealed that the part of the cellular tubulin pool was disassembled and accumulated in the juxtanuclear part of the H1299 cells after the treatment with phenethyl isothiocyanate [19]. Here, we have suggested that the reorganization of β-tubulin may contribute to the alterations in mitotic spindle formation and, in consequence, to the formation of multinucleated giant cells.

Intermediate filaments are also the cytoskeletal components and they consist of many different proteins. One of them is vimentin, which is the major cytoskeletal component of mesenchymal cells [30]. Here, we have documented the accumulation of vimentin around the nucleus in the giant cells and the reorganization of this cytoskeletal protein in the shrunken cells. We have also observed that the reorganization of vimentin after caffeine treatment was connected with the loss of cell-cell contact, what

supports the role of intermediate filaments in maintaining cell shape, cellular migration and cytoskeletal integrity [31,32].

In conclusion, this study shows that exposure of CHO AA8 cell line to caffeine resulted in reorganization of mainly cytoskeletal proteins, such as F-actin, vimentin and tubulin. Furthermore, this alkaloid may induce two type of cell death (apoptosis and mitotic catastrophe) and all elements of cytoskeleton are interconnected and necessary in cell death processes.

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