The protective effect of niacinamide on CHO AA8 cell line against ultraviolet radiation in the context of main cytoskeletal proteins

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online)

Adv Clin Exp Med. 2018;27(3):367-378

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Funding sources

This study was supported by a research task within the framework of the statutory activities and students' research (Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland).

Conflict of interest

None declared

Received on October 7, 2016 Reviewed on October 18, 2016 Accepted on January 5, 2017

Abstract

Background. Niacinamide is a stable and water-soluble form of vitamin B₃, a valuable and versatile cosmetic ingredient, which is well absorbed and tolerated by the skin. A large body of literature has reported on the antioxidant and cell repair properties of niacinamide. Therefore, it has been shown to be useful in the protection of the skin against ultraviolet B (UVB) radiation and free radicals. Despite numerous hypotheses on the mechanism of vitamin B₃, its protective effects have not yet been fully elucidated.

Objectives. The aim of the study was to determine the protective effects of niacinamide on CHO AA8 cell line against UVB radiation. We assessed the following factors: cell death, cell cycle phase distributions, reorganization of main cytoskeletal proteins, such as F-actin, vimentin and β -tubulin, and also alterations at the ultrastructural level.

Material and methods. The material used for our research was Chinese hamster ovary cell line (CHO AA8). We used 4 research groups: 1) control cells; 2) cells treated with niacinamide; 3) cells exposed to UV radiation; and 4) cells co-incubated with niacinamide and next exposed to ultraviolet. The cell death and cell cycle were evaluated by a Tali[®] based-image cytometer. A fluorescence microscope was used to assess the reorganization of cytoskeletal proteins, whereas a transmission electron microscope enabled the evaluation of the alterations at the ultrastructural level of cells.

Results. We showed that UV-induced apoptosis and cell cycle distributions during treatment with niacinamide resulted in a non-statistical significance in cell survival and no significant changes in the morphology and cytoskeleton in comparison to the control group. In turn, a combination of both factors led to an increase in the population of live cells and a decreased level of apoptotic cells in comparison to UV-exposed cells.

Conclusions. Our results confirmed the harmful effects of UV radiation on CHO AA8 cell line. Furthermore, niacinamide can protect cells against these factors, and the mechanism of action may be related to the stabilization of the cell cytoskeleton.

Key words: UV radiation, F-actin, vimentin, niacinamide, β-tubulin

DOI

10.17219/acem/68289

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Introduction

Vitamin B₃, also known as niacin (nicotinic acid vitamin), is found in several forms, including niacinamide (nicotinamide; Niac). Furthermore, as niacin cannot be synthesized by the human body, it has to be delivered to the organism from the environment. It can be found in many foods, such as green vegetables, beans, milk, eggs, yeast, and meat or fish. Vitamin B₃ is involved in the mechanisms of DNA repair and genomic stability. Furthermore, niacin deficiency results in an increased rate of somatic mutations and toxicity caused by reactive oxygen species (ROS), which is associated with an increased risk of cancer and its elevated progression rate. 1,2 Niacinamide is an amide form of vitamin B₃, and it is also known as the precursor of important cofactors: niacinamide adenosine dinucleotide (NAD+) and its phosphate derivative – niacinamide adenosine dinucleotide phosphate (NADP).3 These cofactors and their reduced forms (NADH and NADPH) are involved in more than 40 cellular biochemical reactions, including cellular biochemistry and energy metabolism, and in the mechanisms of cell protection against ROS.1 From the clinical point of view, nicotinic acid and niacinamide are useful in the treatment of various types of diseases. It has been shown that high doses of nicotinic acid may be potent in hypercholesterolemia treatment due to the reduction of lipids, e.g., low density lipoprotein (LDL), fatty acids (FAs) or cholesterol, as well as by the increase of high density lipoprotein (HDL).^{1,4} On the other hand, niacinamide is a clinically attractive pharmacological agent for the prevention of type 1 diabetes mellitus due to the protection of islets.⁵ Likewise, Feng et al. indicated that nicotinamide may be used in the treatment of cardiac diseases and strokes as well as fetal alcohol syndrome (precursor of Bacterial NAD+).6 Moreover, niacinamide, through an increase of intercellular NADP levels, improves and stabilizes the skin barrier, and also shows strong antioxidant properties.7 The effect on the epidermal barrier function is probably associated with stimulating the differentiation of keratinocytes and/or the upregulated synthesis of ceramide and keratins. The above-mentioned factors and durability, water solubility and good penetration through the stratum corneum are the reasons why it is also widely used in sunscreens and anti-aging cosmetics.8

Ultraviolet radiation (UV) is one of the most common factors leading to skin cancer and photoaging. It is one of the major sunlight wavelengths on the light spectrum and it has been divided into 3 sections: UVC, UVB and UVA. UVC (wavelengths: 100–180 nm) is effectively blocked from reaching the Earth's surface by the stratospheric ozone layer of the atmosphere. As the reports indicate, exposure to UV leads to skin burns and carcinogenesis. It is widely used in germicidal lamps. UVB (wavelengths: 280–315 nm) reaches the Earth's surface in approx. 1–10% and induces unfavorable consequences to eyes and the

skin. However, it is involved in the synthesis of vitamin D_3 . Furthermore, the waves of UVB are absorbed by the skin with the consequence of sunburns, tanning, wrinkling or photoaging, which lead to skin cancer. UVA is not blocked by the stratospheric ozone layer and reaches the Earth's surface in approx. 90–99%. As UVA is characterized by long wavelengths (315–400 nm) and low energy, it can penetrate deeper into the skin than other UV types. It causes photoaging and leads to skin cancer. However, UVA is used in medicine, in the treatment of psoriasis, and in solariums.⁹

Due to that, niacinamide is known as an anti-aging agent and it is characterized by many beneficial prosperities. We examined its potential protective effect against ultraviolet radiation on an in vitro cell model of fibroblasts. To our knowledge, this study for the first time demonstrated the influence of niacinamide on the main cytoskeletal proteins such as F-actin, vimentin and β -tubulin in CHO AA8 cells. Moreover, we suggested that alterations of the cytoskeletal structure can be crucial to the protective effect of this compound.

Material and methods

Cell cultures and experimental treatments

The Chinese hamster ovary cell line (CHO AA8), kindly provided by Prof. M.Z. Zdzienicka (Department of Molecular Cell Genetics, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland), was routinely grown in Minimum Essential Medium (MEM, Lonza Group AG, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS, Gibco/Invitrogen Life Technologies, Carlsbad, CA, USA), 50 µg/mL gentamycin (Sigma-Aldrich Co., St. Louis, USA) and 1% non-essential amino acids (Sigma-Aldrich). Cells were cultured in 5% CO₂, in a humidified atmosphere of 95% air, at 37°C. For further experiments, after 80% confluence, the cells were removed by trypsin (Sigma-Aldrich) and subcultured on 6- or 12-well plates. After 24 h culture, the cells were treated with niacinamide (Sigma-Aldrich) at a final concentration of 1 and 10 mM to the cell medium, or exposed to ultraviolet radiation for 5 and 15 min. The doses of niacinamide were chosen based on the literature, while the time of UV exposure was previously described by us as inducing visible alterations in cells. 10,11 In order to check the combination of both factors, first, CHO AA8 cells were preincubated with niacinamide for 24 h, and next the medium was partially removed and the cells were exposed to an indicated dose of ultraviolet light at a wavelength of 254 nm (UV). After exposure, the incubation was continued with fresh medium and cultured for 24 h for the next experiments. The control cells were grown at identical conditions without treatment with niacinamide and/or UV radiation.

Based-image cytometry assay

In order to determine cell death, the cells were stained with propidium iodide (PI) and Annexin V-Alexa Fluor® 488 (Tali® Apoptosis Kit – Annexin V-Alexa Fluor® 488 & Propidium Iodide, Invitrogen, Thermo Fisher Scientific, Waltham, USA) according to the protocol previously described.¹² The cell pellets were resuspended in an Annexin Binding Buffer (ABB) and incubated with 5 µL of Annexin V-Fluor® 488 (Invitrogen, Thermo Fisher Scientific) in the dark for 20 min. Then, the cells were centrifuged at 300 g \times 5 min and washed with ABB, whereupon 1 μ L of propidium iodide was added for 4 min (incubation in the dark). Finally, the stained cells were loaded into a Tali® Cellular Slides (Invitrogen, Thermo Fisher Scientific) and analyzed with a Tali® based-image cytometer (Invitrogen, Thermo Fisher Scientific) in accordance with the instruction manual. The results were analyzed on the assumption that the viable cells were characterized by negative signal for PI and Annexin V; apoptotic cells represent early and late apoptosis (all with positive signal for Annexin V-Alexa Fluor 488); necrotic cells were only PI-positive.

We measured cell cycle distribution using propidium iodide and a Tali® based-image cytometer (Invitrogen, Thermo Fisher Scientific). The CHO AA8 cells were harvested by trypsin and fixed with ice-cold 70% ethanol in distillated water, and stored at -20° C for a few days. Afterwards, the cells were centrifuged at 650 g × 7 min, and were then rinsed with 1 mL of cold phosphate buffered saline (PBS) and centrifuged at 500 g × 5 min. Subsequently, the cell pellets were resuspended in Tali® Cell Cycle Kit (Invitrogen, Thermo Fisher Scientific) and incubated in the dark for 30 min. The Tali® cell cycle program was used to determine the fractions of cells in the sub-G1, G2/M, S, G0/G1 phases from the cell cycle distribution.

Each experiment was repeated in triplicate, and the results were analyzed using FCS Express 4 Plus Researches Edition (deNovo Software, v. 4.03; Glendale, CA, USA).

Fluorescence staining of cytoskeletal proteins

To understand the protective mechanism of niacinamide, alterations in the main cytoskelatal proteins were analyzed. The CHO AA8 cells were cultured on the coverslips. First, in order to perform fluorescence staining of β-tubulin (microtubules), the cells were fixed in 3,3′-dithiodipropionic acid (DTSP) (Sigma-Aldrich Co.), diluted in microtubule-stabilizing buffer (MTSB) (1 mM EGTA, 10 mM PIPES, 4% polyethylene glycol); Sigma-Aldrich Co.; 1:50, 10 min at room temperature (RT), and were washed with DTSP in 0,5% Triton X-100 in MTSB (TSB) (1:50, 10 min, RT). Next, the cells were permeabilized with Triton-X 100 (Serva, Heidelberg, Germany) in TSB (10 min, RT) and incubated with 4% paraformaldehyde (PFA) (Serva) in HBSS (Hanks' Balanced

Salt Solution, Sigma-Aldrich Co.) for 20 min at RT. Then, the CHO AA8 cells were rinsed in PBS (3×5 min, RT) and blocked in 1% bovine serum albumin (BSA)-PBS (15 min, RT). In turn, for fluorescence staining of vimentin and F-actin (intermediate filaments and microfilaments, respectively), the material was fixed in 4% PFA (20 min, RT), rinsed with PBS (3 × 5 min, RT) and blocked in 1% BSA in PBS (15 min, RT). Afterwards, the CHO AA8 cells were incubated with the primary antibody special for vimentin (Sigma-Aldrich) and β-tubulin (Sigma-Aldrich) at a dilution 1:60 and 1:80 in 1% BSA-PBS, respectively (1 h, RT). After the series of washes with PBS (3 \times 5 min, RT), the secondary antibody tetramethylrhodamine (TRITC) anty-mouse IgG (Sigma-Aldrich Co.) was used (1:85 in PBS, 1 h, RT). In order to visualize microfilaments (F-actin), the cells were incubated with Alexa Fluor 488® conjugated with phalloidin (Invitrogen, Thermo Fisher Scientific, Molecular Probes; 1:40 in PBS, 20 min, RT). Next, the nuclei of cells were stained by 4',6-diamidyno-2-fenyloindol (DAPI) (Sigma-Aldrich Co.; 1:20000 in PBS, 10 min, RT) and after the final rinse with PBS, the slides were mounted in Aqua-Poly/Mount (Polysciences Inc., Warrington, USA). The fluorescence analysis was performed using the Nikon Eclipse E800 fluorescence microscope (Nikon, Tokyo, Japan) and NIS-Elements 4.0 software (Nikon).

Assessment of ultrastructural alterations

In order to assess the ultrastructural alterations, a transmission electron microscope was used. After the cells were cultured, the material was fixed with 3.6% glutaraldehyde (Polyscience, Warrington, PA, USA) in 0.1 M sodium cacodylate buffer (Roth, Karlsruhe, Germany) for 30 min at RT and rinsed with 0.1 M cacodylate buffer (pH 7.4). Then, following the use of 1% osmium tetroxide in cacodylate buffer (1 h, RT), the CHO AA8 cells were washed with 0.1 M cacodylate buffer and dehydrated through a graded series of alcohols (30-90%) and acetone (90-100%). Subsequently, the study material was embedded in Epon 812 (Roth), and polymerization of the resin occurred for a few days (24 h at 37°C and 120 h at 65°C). Afterwards, the parts of the material selected were cut into ultra-thin sections using a Reichert Om-U3 ultra-microtome (Vienna, Austria), placed on copper grids and stained with 1% uranyl acetate. The prepared material was examined using a JEM-100CX electron microscope (Jeol, Tokyo, Japan).

Statistical analysis

Statistical comparisons between the 2 groups of cell death and cell cycle data were performed using a 2-tailed Mann-Whitney U test. Differences between the groups were considered significant when p < 0.05. GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses.

Results

Effect of niacinamide and/or UV on cell death

We studied the occurrence of cell death of CHO AA8 cells with treatment by various concentrations of niacinamide, different time of exposure to UV, and their combination. The analysis of cell death was performed using a Tali® Image-based cytometer, following Annexin V-Alexa

Fluor 488® and Propidium Iodide double staining. The representative plots were presented in Fig. 1 A and the mean percentage of results is presented in Table 1. In the CHO AA8 cells, no statistically significant differences in the percentage of live and apoptotic cells were observed following the treatment with all niacinamide doses as compared to the control. However, the exception was statistically significant increase in the percentage of Annexin V-positive cells after incubation with niacinamide at 10 mM concentration. In turn, the data demonstrated that

Table 1. The mean percentage of cell death results

Cells groups	CTRL 24h	1 mM Niac	10 mM Niac	5 min UV	15 min UV	1 mM Niac/ 5 min UV	1 mM Niac/ 15 min UV	10 mM Niac/ 5 min UV	10 mM Niac/ 15 min UV
IP-/A-	95.63	93.68	93	87.01*	75.08*	93.49	91.44	93.87	87.89*
IP-/A+	1.78	2.57	5.19*	10.61*	25.5*	5.29*	5.8*	2.31	3.92
IP+/A-	2.01	2.53	2.14	3.58	1.41	3.95	4.11	2.36	7.72*

^{*} Statistically significant results in comparison to the control (p < 0.05); CTRL – control; Niac – niacinamide; UV – ultraviolet.

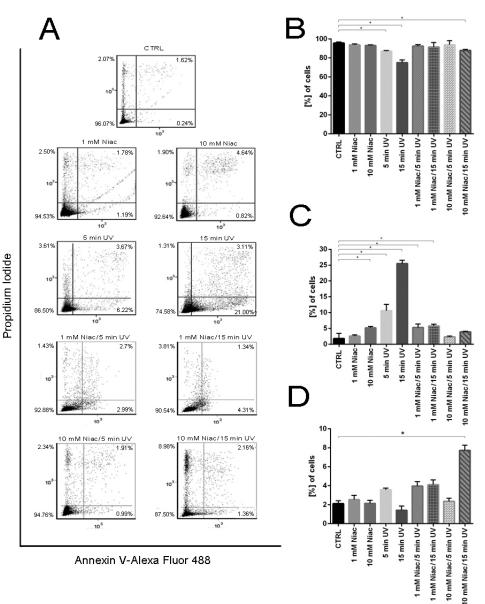


Fig. 1. Based-image cytometric analysis of cell death using Annexin V-Alexa Fluor 488 and Pl assay

A – representative plots;

- B the percentage of viable cells;
- C the percentage of apoptotic cells;
- D the percentage of necrotic cells. The cells were treated with different concentration of niacinamide (1 and 10 mM) for 24 h, exposed to 5 and 15 min UV, and with both factors

(1 and 10 mM) for 24 h, exposed to 5 and 15 min UV, and with both factors combined; asterisks indicate statistically significant differences from control cells (p < 0.05).

ultraviolet radiation decreased the mean percentage of viable cells in time-dependent manner from 87.01% for 5 min to 75.08% for 15 min. Besides, we showed that the population of apoptotic cells ranged from 10.61% following 5 min of UV radiation to 25.5% after 15 min exposure to UV. In addition, the CHO AA8 cells exposed to ultraviolet irradiation resulted in a statistically significant decrease of cell viability, thereby leading to the statistically significant increase of apoptotic cells in comparison to untreated cells (Fig. 1 B–D).

As shown in Fig. 1 B, after combined treatment with niacinamide and UV radiation, we did not observe statistically significant differences of cell viability in comparison to control, except for 10 mM Niac/15 min UV, whereas the mean percentage of live cells was 87.89%. Furthermore, following treatment with 1 mM Niac/5 min UV and 1 mM Niac/15 min UV, the Annexin V staining showed statistically significant differences in the population of apoptotic cells in comparison to untreated cells (from 1.78% for control to 5.29% and 5.8%, respectively). The data demonstrated that preincubation with increasing doses of niacinamide and exposure for UV resulted in a statistically significant increase in the mean percentage of viable cells (excepting 10 mM Niac/5 min UV) in comparison to the results following only UV radiation, thus reducing the Annexin V-positive population of cells (Fig. 1 B, C).

Cell cycle redistribution following the treatment with niacinamide and/or UV

During the project, the effect of the treatment with niacinamide and/or UV radiation on the cell cycle of CHO AA8 cell line was examined. Propidium iodide (PI) staining and an image-based cytometric analysis were used to determine cell cycle distribution and the results are shown in Fig. 2 and Table 2. Our data presented that the incubation with 1 mM and 10 mM niacinamide led to an increase in the cell population of sub-G1 phase (Fig. 2 A). In turn, proportions of cells in sub-G1 phase increased from 4.83% (control) to 12.78% (5 min) and 29.01% (15 min) following UV radiation in a time-dependent manner. As regards the population of cells in sub-G1, after co-incubation with chosen doses of niacinamide and exposure to UV, there was a statistically significant increase in comparison with the population of control cells (Fig. 2 A). Nevertheless, we observed a statistically significant decrease in the mean percentage of cells classified as G2/M in comparison with the control as a consequence of treatment with all doses of niacinamide, both the exposure times of ultraviolet radiation and all combinations of these factors (Fig. 2 B). Concurrently, there were non-statistical differences in the population of cells categorized as S phase when compared with untreated cells excepting the cells treated with 1 mM niacynamide and exposed to UV light for 15 min (decrease

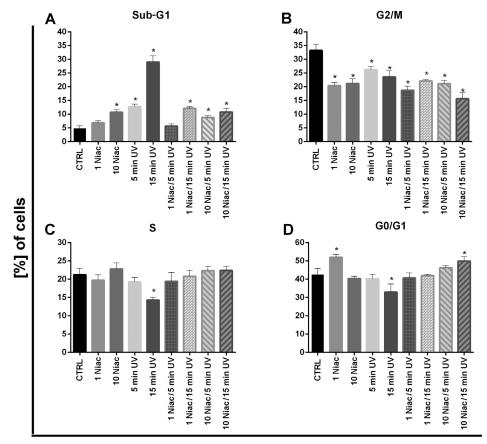


Fig. 2. Based-image cytometric analysis of cell cycle distribution in CHO AA8 cells treated with different concentrations of niacinamide (1 and 10 mM) for 24 h, exposed to 5 and 15 min of UV, coincubated with Niac, and next exposed to UV

The percentage of cells in: A – sub-G1 phase; B – G2/M phase; C – S phase; D – with G0/G1 DNA content; asterisks indicate statistically significant differences from control cells (p < 0.05).

doses [mM] and time of exposure [min]

Table 2. The mean percentage of cell cycle distributions

Cells groups	CTRL 24 h	1 mM Niac	10 mM Niac	5 min UV	15 min UV	1 mM Niac/ 5 min UV	1 mM Niac/ 15 min UV	10 mM Niac/ 5 min UV	10 mM Niac/ 15 min UV
Sub-G1	4.83	6.86	10.73*	12.78*	29.01*	5.64	12.10*	8.79*	10.83*
G2/M	33.24	20.42*	21.21*	26.24*	23.63*	18.74*	22.10*	21.13*	15.64*
S	21.26	19.77	22.79	19.27	14.32*	19.45	20.79	22.33	22.40
G0/G1	42.57	51.98*	40.44	40.21	32.97*	40.79	41.98	46.29	49.92*

^{*} Statistically significant results in comparison to the control (p < 0.05); CTRL – control; Niac – niacinamide; UV – ultraviolet.

to 14.32%) (Fig. 2 C). Similarly, a statistically significant decrease in the proportions of cells in G0/G1 phase was detected after 15 min of ultraviolet radiation. On the other hand, we also observed that the CHO AA8 cells treated with 1 mM Niac and 10 mM Niac/15 min UV were characterized by a statistically significant increase in G0/G1 phase population. Moreover, as shown in Fig. 2 D, the median percentage of cells in G0/G1 was at a similar level as in the case of control cells.

Fluorescence staining of the main cytoskeletal proteins

Morphological changes of cytoskeleton architecture depended on the times and doses of factors. The control cells were characterized by oval nuclei, regular shape, size and numerous actin stress fibers, typical for fibroblasts (Fig. 3 A). After 24 h of niacinamide treatment, the cells

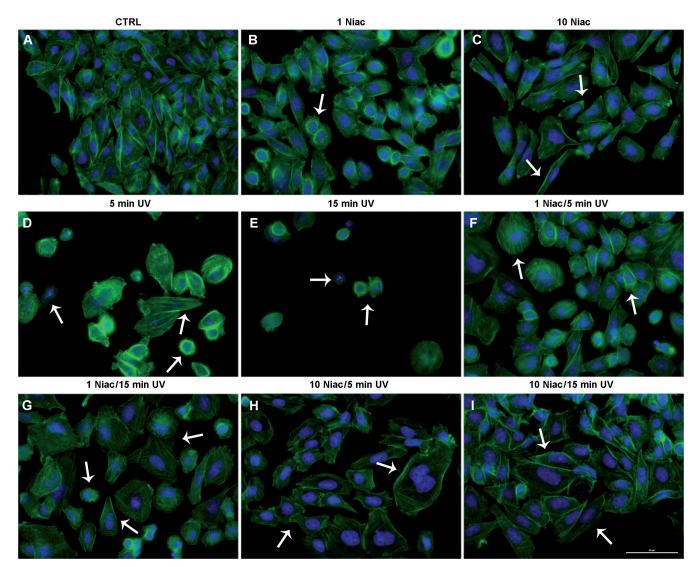


Fig. 3. CHO AA8 cells stained for F-actin; cell nuclei labeled with DAPI

A – control cells; B – cells treated with 1 mM of niacinamide; C – cells treated with 10 mM of niacinamide; D – cells exposed to 5 min UV; E – cells exposed to 15 min UV; the combination of both factors: F – 1 mM Niac/5 min UV; G – 1 mM Niac/15 min UV; H – 10 mM Niac/5 min UV; I – 10 mM Niac/15 min UV; Bar = 50 μ m.

Adv Clin Exp Med. 2018;27(3):367-378

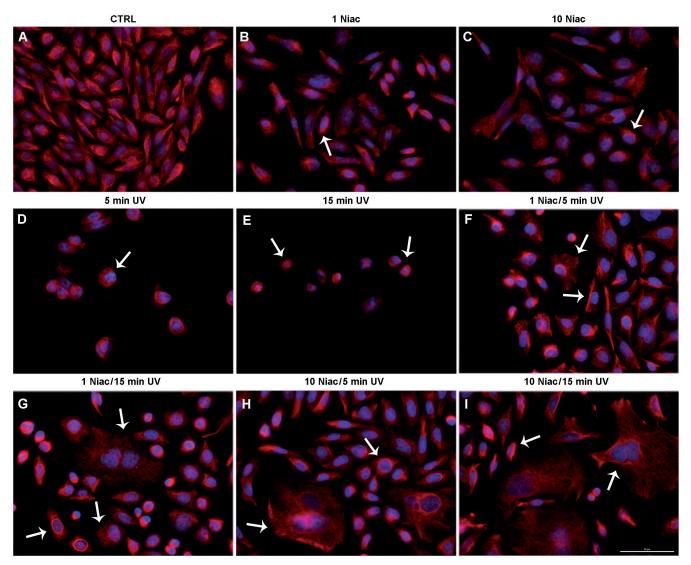


Fig. 4. CHO AA8 cells stained for vimentin (intermediate filaments); cell nuclei labeled with DAPI

A – control cells; B – cells treated with 1 mM of niacinamide; C – cells treated with 10 mM of niacinamide; D – cells exposed to 5 min UV; E – cells exposed to 15 min UV; the combination of both factors: F – 1 mM Niac/5 min UV; G – 1 mM Niac/15 min UV; H – 10 mM Niac/5 min UV; I – 10 mM Niac/15 min UV; Bar = 50 μ m.

possessed actin filaments in the perinuclear area and intensive fluorescence of F-actin in the cortical part of the cell, like in untreated cells (Fig. 3 B, C). In turn, according to our previous results (cell death analysis), we noticed shrunk cells with depolymerization of the actin network following UV irradiation. After 5 min of ultraviolet exposure, F-actin formed a ring-like structure around the nucleus. However, in the enlarged cells, the actin network was expanded (Fig. 3 D). Following exposure of 15 min, strong F-actin rearrangement was observed in most of shrunk cells (Fig. 3 E). The assessment of F-actin structure showed that in the case of following groups – 1 mM Niac/5 min UV and 1 mM Niac/15 min UV numerous stress fibres were clearly visible (Fig. 3 F, G). The cells co-treated with higher concentration of niacinamide (10 mM) and next exposed to UV had a voluminous actin network and contained more bundles of stress fibers, especially in the enlarged cells (Fig. 3 H, I). Furthermore, fluorescence staining of microfilaments showed their accumulation in the cortical part and at the border between neighboring cells (Fig. 3 F–I).

In turn, in control cells, vimentin and microtubules were well-developed and were characterized by regular scaffold of thin filaments (Fig. 4 A, 5 A). Our cytoskeletal studies revealed that after exposure of the CHO AA8 cells to niacinamide, a network of intermediate filaments was regular and there appeared some cells with intensity labeling of vimentin in the nuclear area (Fig. 4 B, C). Similar to the observation of F-actin, the image of 5 and 15 min exposure of UV showed the large population of apoptotic cells which were characterized by rounded shape, shrunk nuclei and strong reorganization of vimentin bundles (Fig. 4 D, E). Following treatment with combination of all doses of niacinamide and both the exposure times of UV irradiation, our observation revealed that part of cells possessed thick vimentin bundles in perinculear region (Fig. 4 F–I).

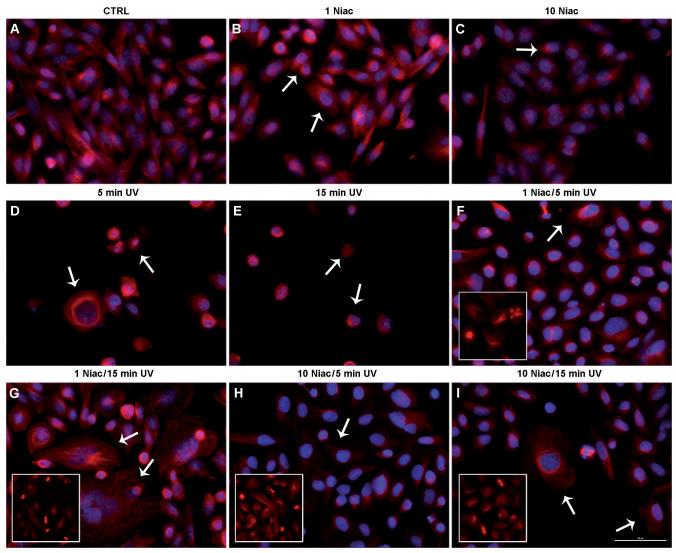


Fig. 5. CHO AA8 cells stained for β -tubulin (microtubules); cell nuclei labeled with DAPI

A – control cells; B – cells treated with 1 mM of niacinamide; C – cells treated with 10 mM of niacinamide; D – cells exposed to 5 min UV; E – cells exposed to 15 min UV; the combination of both factors: F – 1 mM Niac/5 min UV; G – 1 mM Niac/15 min UV; H – 10 mM Niac/5 min UV; I – 10 mM Niac/15 min UV; Bar = 50 μ m.

Furthermore, we also observed the small population of shrunken cells with strongly labeled of vimentin in the nucleus area and some enlarged cells, where the network of intermediate filaments were strongly extended (Fig. 4 G–I).

As shown in Fig. 5 (β -tubulin staining), following treatment with different concentrations of Niac, the population of altered cells was very small. Some CHO AA8 cells were characterized by strongly labeled β -tubulin localized in the perinuclear region, and some enlarged cells presented thick microtubules (Fig. 5 B, C). Additionally, we observed that UV radiation promoted changes in the distribution of main cytoskeletal protein such as β -tubulin in the CHO AA8 cells. The shorter time of UV exposure led to a high concentration of microtubules as ring-like structure in the perinuclear area. Furthermore, the cells presenting thick β -tubulin were occasionally also observed. Besides, fluorescence microscopic observation of microtubules demonstrated that the longer time of UV radiation (15 min) resulted in reorganization

of β -tubulin, especially in apoptotic cells (Fig. 5 D, E). An interesting fact worth emphasizing is that after exposure of the CHO AA8 cells to all combinations of chosen factors, we observed a cell population with a visible mitotic spindle (Fig. 5 F–I inserts). Moreover, as shown in Fig. 5 F–I, a strongly expanded network of tubulin with thick bundles was noticed in most of the enlarged cells. After the higher dose of Niac (10 mM) and time of UV exposure (15 min), the higher labeling of β -tubulin in the perinuclear region was seen (Fig. 5 I).

Furthermore, compared to untreated CHO AA8 cells, treatment with both niacinamide and UV radiation caused the alterations in cell shape from spindle type, typical for fibroblasts, to more rounded and flattened. Also, microscopic observation revealed a smaller population of cells with hallmarks characteristics for apoptosis, such as shrinkage of the cells or chromatin condensation, than in the case of ultraviolet exposure alone.

Alterations at the ultrastructural level

The last step of our studies was the ultrastructure analysis of the CHO AA8 cells cultured with niacinamide, exposed to different time of UV and treated with both factors. The alterations were performed using a transmission electron microscope (TEM) and these methods confirmed our previous observations. Namely, the control cells possessed a regular shape and an oval single nucleus (Fig. 6 A). TEM revealed that treatment with niacinamide resulted in the presence of some giant cells and cells with swollen mitochondria or a small amount of vacuoles, probably cantained fine fibrillar material. Nevertheless, a population of these cells did not show significant morphological differences in comparison with the untreated cells (Fig. 6 B, C). In turn, following the exposure to ultraviolet radiation, the cells were characterized by visible morphological changes in the nucleus area and cytoplasm. After 5 min of UV radiation, the alterations were related to the marginalization of chromatin and segmented/lobulated nuclei (Fig. 6 D). Besides, some UV-irradiated cells (15 min) presented acute damage manifestation and they included a degenerated cytoplasm and/or extensive cytoplasmic vacuolization, swollen and disturbed mitochondria, and shrunk nuclei with a high condensation of chromatin (Fig. 6 E). On the other hand, electron micrographs of the CHO AA8 cells treated with both niacinamide and UV rays showed no dramatic changes in size and shape of cells in comparison to the population of untreated cells. We only observed an irregular shape of nucleus (segmented) with visible nucleolus, especially in the cells following 1 mM Niac/15 min UV and 10 mM Niac/5 min UV (Fig. 6 G, H), whereas, as showed in Fig. 6 F, I, significant changes in the ultrastructure of the CHO AA8 cells were not clearly observed.

Discussion

UV radiation is involved in many different harmful processes in cells. It has been reported that UV exposure results in the formation of cyclobutane-pyrimidine dimers (CPDs) and 6–4 photoproducts (6–4 PPs), which evoke the most abundant cytotoxic and mutagenic DNA lesions.¹³ Rastogi et al. have described a number of biological effects

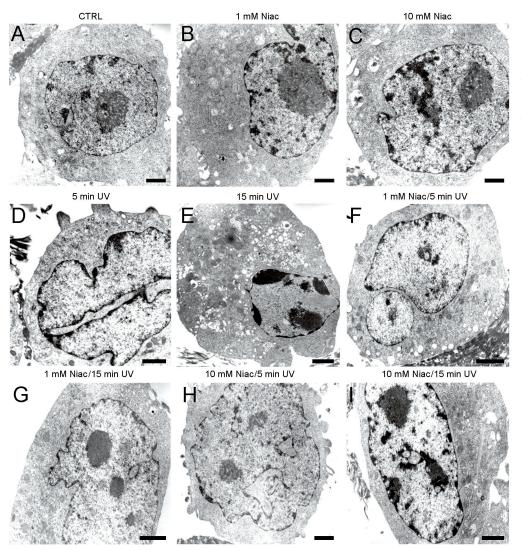


Fig. 6. Transmission electron micrographs of CHO AA8 cells

A – electron micrographs of control cells; B – cells treated with 1 mM of niacinamide; C – cells treated with 10 mM of niacinamide; D – cells exposed to 5 min UV; E – cells exposed to 15 min UV; the combination of both factors: F – 1 mM Niac/5 min UV; H – 10 mM Niac/15 min UV; I – 10 mM Niac/5 min UV; Bar = 5 μ m.

of UV radiation, including alterations in proteins or DNA, N_2 fixation, as well as a reduction in cell growth and division. Furthermore, Britt showed that UV radiation induces DNA damage and alterations in replication and transcription, leading to deletions of nitrogenous basis. Moreover, UV radiation results in the induction of oxidative stress, and it is involved in skin carcinogenesis. The research presented here was performed on one of the experimental models commonly used in many biological, pharmaceutical and medical studies – Chinese hamster ovary cell line (CHO AA8).

According to the literature, vitamins are necessary for proper cell functioning, development, and metabolism in each organism. It is known that the group of 13 vitamins, including vitamin A, C, B, K, and D has many beneficial features and probably prevent cancers and various diseases, such as cardiac disorders, arthritis and diabetes. This is why a diet rich in vegetables and fruits, and hence rich in many vitamins, is an important factor in maintaining good skin condition. In particular, vitamins A, B, C, and E exert an effect on moisture, spots, redness, and wrinkles of the skin. Among them, niacinamide (an amide form of vitamin B₃) has been described as an agent that can improve skin condition stabilize epidermal barrier function and inhibit photocarcinogenesis.^{2,8}

In the present study, we reported that niacinamide specifically induces reorganization of main cytoskeleton proteins such as F-actin, vimentin and β-tubulin, resulting in the protection against UV irradiation-induced apoptosis in CHO AA8 cell line. We found that UV radiation results in the decrease of cell survival and the increase in the percentage of apoptotic cells. Similar data was presented by Grzanka et al., where the CHO AA8 cells were irradiated by UVC for 2 min at a fluency of 100 J/m. They also observed morphological changes typical for programmed cell death.¹⁷ It has also been reported that UV radiation results in cell death in mammalian cell lines, including non-small cell lung cancer (A549), breast cancer (MCF7), human kidney cells (293), HeLa cells, and keratinocytes. 11,18-21 What is more, the data presented here showed that the treatment of CHO AA8 cell line with different concentrations of niacinamide did not induce the increase in cell population characterized by hallmarks typical for apoptosis. According to Knip et al., compounds of niacin, such as niacinamide, are non-toxic, while Iwata et al. suggested that the amides of vitamin B may promote differentiations in HL-60.1,22,23 We presented here that the combination of niacinamide and exposure to UVB resulted in the decrease of the number of apoptotic cells as compared to UV-irradiated CHO AA8 cells. Surjana et al. suggested that nicotinamide (= niacinamide) possesses protective properties against ultraviolet radiation. In their study on human keratinocytes HaCaT and ex vivo skin pre-treated with nicotinamide and exposed to ssUV (UVA + UVB), they demonstrated that these forms of niacin enhanced the repair of UV-induced DNA damage through the reduction of CPDs and 80xoG.^{3,24} Furthermore, other studies showed that nicotinamide prevented immune suppression (human and mice) and cellular energy loss (human keratinocytes) induced by ultraviolet irradiation, and also by carcinognesis (mice).^{3,25}

Here, we confirmed that ultraviolet irradiation induces apoptosis, which was manifested by the higher percentage of sub-G1 cells population and the lower percentage of cells with DNA content corresponding to S phase. Several other studies also revealed that UV results in G2 arrest of cell cycle in human keratinocytes, human skin fibroblasts W1 and in SV40 cells. ^{26,27} Surjana et al. reported that amides of niacin did not affect cell division. ³ The data presented here also shows that nicotinamide did not cause significant changes in CHO AA8 cell cycle distributions.

Despite numerous hypotheses, the protective mechanisms of niacinamide action against UV radiation are still not well understood. The knowledge of the cytoskeleton has been expanding for several decades, and it is known that this structure plays a critical role in almost all cellular processes. In our study, we observed that UV radiation led to the reorganization and degradation of F-actin, β-tubulin and vimentin. Similar changes in the microfilament network were described by Veselká and Janisch, Grzanka et al. and Klimaszewska-Wiśniewska et al. They also noticed that UV-irradiated cells were characterized by the presence of F-actin aggregates and high-intensity fluorescence of actin fibers, especially in the perinuclear regions in shrunk cells. 11,17,28 Desozua et al. suggested that the alterations in actin cytoskeleton may be a reference to the assessment of apoptosis.²⁹ Furthermore, the disturbances of microfilament reorganization may result in incorrect cells adherence, and then lead to apoptosis, which in our experiments was confirmed by the presence of rounded cells population and a higher percentage of apoptotic cell after irradiation by UV.30 Veselká and Janisch presented the effect of UV radiation on the microtubule network in fibroblast L929 cell lines and showed its reduction, thickening and fragmentation.^{28,30} Our results also showed restricted distribution of β -tubulin in UV-irradiated cells. Moreover, Grzanka et al. demonstrated that UV exposure results in disassembly in microtubules and condensation of intermediate filaments in the perinculear area in other cell lines.¹⁷ Here, after UV irradiation, CHO AA8 cells also possessed the depolymerized vimentin, most strongly located in the nuclear region. Our studies confirmed the negative effect of UV on morphology and cytoskeletal proteins, and we suggest that these changes lead to cell death. Based on our knowledge, there are no similar papers on the effect of niacinamide as a factor applied alone and in combination with UV on Chinese hamster ovary cell line, especially in the context of cytoskeleton network. In the present study, we showed that niacinamide-treated cells did not show significant differences in cytoskeletal structures as compared to untreated cells. Moreover, we noticed that after pre-treatment with niacinamide and

then exposure to UV, the CHO AA8 cells seemed to be more flattened and thus more attached to the surface in comparison to UV-irradiated cells. This was confirmed by the fact that we observed intensive fluorescence of microfilaments in the cortical area in cells, which suggests enhanced adherent properties of the cells. Furthermore, we noticed a higher number of stress fibers and enlarged cells characterized by an extensive, bold network of cytoskeletal proteins. In addition, the presence of strongly labeled vimentin in ring-like form localized in the perinuclear region may augment filament assembly to build new intermediate filaments, and hence strengthen the network.³¹ Moreover, we showed that pre-treatment with niacinamide and then exposure to UV promotes cell growth and division in the form of visible mitotic figures and similar percentages of cells in S phase. In summary, we suggest that the amide of vitamin B₃ induces the stabilization of microfilaments and intermediate filaments.

Our transmission electron microscope results presented cells which were characterized by visible condensation of chromatin, nucleus segmentation, vacuolization of cytoplasm, or swollen mitochondria after ultraviolet irradiation. Likewise, Grzanka et al. showed that Chinese hamster ovary cells after exposure to UV irradiation exhibited similar alterations.¹⁷ Furthermore, other authors described the appearance of alterations in different UV-treated types of cell lines such as non-small lung cancer A549 and melanoma WM35 cells lines. 11,32 On the other hand, niacinamide-treated cells predominantly showed essentially normal ultrastructure; in addition, swollen mitochondria were sporadically presented. Similar results were reported by Petrali et al., who described the protective effect of niacinamide on human lymphocytes and showed that the chosen amide of vitamin B₃ did not cause significant changes at the ultrastructural level as compared to the control.³³ The results presented here showed that the treatment with a combination of both factors (niacinamide and UV) induced visible changes in the nucleus area and less destruction of the cytoplasm as compared to ultraviolet irradiated CHO AA8 cells. In this paper, we described for the first time the influence of niacinamide and its combination with UV on the ultrastructural changes in the CHO AA8 cells.

It has been shown that vitamin B₃ protects human keratinocytes against UV irradiation. Lin et al. have shown that co-incubation with niacin enhances the pro-survival pathway (through mTOR, AKT, S6) in HaKaT cells, and thus protects against cell death induced by UV.³⁴ Moreover, the effect of niacinamide on the primary melanocytes cell line has been reported by Thomson et al. They suggested that this form of vitamin B enhances the repair of DNA damage.²⁴ Several other studies described the protective properities of niacinamide.^{3,7,33,35,36} Nevertheless, the data presented here allows us to assume that niacinamide protects CHO AA8 cells against ultraviolet exposure.

In conclusion, we described for the first time the influence of niacinamide on cytoskeletal proteins such as Factin, vimentin and β -tubulin, also at the ultrastructural level in Chinese hamster ovary cell line. Moreover, our results also confirmed the harmful effects of UV radiation on chosen experimental material. Data presented in this paper may provide new information on niacinamide in the context of protection properties. Furthermore, we suggested that this compound can protect cells against ultraviolet irradiation, and the mechanism of action may be related to the stabilization of cell cytoskeleton. These results are very important for the development of primary biological sciences, because the full mechanism of niacinamide is still unexplained.

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