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THE PROAPOPTOTIC INFLUENCE OF $AgNO_3$ ON HUMAN KERATINOCYTES AND FIBROBLASTS *IN VITRO*, THE IMPACT FOR BURNED PATIENT MANAGEMENT

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Abstract: It was shown that ointments containing silver compounds delay wound healing and increase the risk of hypertrophic scarring in burned patients. The aim of the study was to establish the influence of $AgNO_3$ on cell viability and apoptosis of keratinocytes and fibroblasts *in vitro*. Foreskin was used to establish primary human keratinocyte and fibroblasts cell cultures. Keratynocytes grew in DMEM/Ham's-12 at 3:1 ratio, supplemented with 10% FBS, EGF, insulin, transferin, triiodothyronine and hydrocortisone. Fibroblasts were cultured in DMEM with addition of 10% FBS. The influence of $AgNO_3$ on keratinocyte and fibroblast cultures was evaluated by fluorescence microscopy and flow cytometry. Double staining with Annexin V-FITC and propidium iodide was performed. The $AgNO_3$ at lower concentration (3 and 15×10^{-4}M/dm^3) than used for patient's treatment (31×10^{-4}M/dm^3) revealed to be toxic and trigger apoptosis in human keratinocytes and fibroblasts. The ointments containing nitrates should be used with caution especially in conditions where the epithelial layer is destroyed. The nitrate can negatively affect wound healing.

Keywords: wound healing, wound dressing, in vitro toxicology, silver ions, apoptosis

The conservative treatment of burned wounds consists initially in the prevention of infections and drying, as these are the two main factors responsible for delayed wound healing (1). A prolonged healing period leads to hypertrophic scar formation. Clinical practice shows that the use of allografts prevents wound drying and infections, therefore promoting epithelialization (2). The use of dressings containing 0.5% (31×10^{-4} M/dm^3) $AgNO_3$ or ointments containing silver compounds has been shown to prevent mainly *Pseudomonas aeruginosa* infection, but at the same time delaying the healing process and increasing the risk of hypertrophic scarring (2, 3). $AgNO_3$ is toxic to cultured human and mouse fibroblasts (4, 5). The above observations have encouraged authors to research the influence of silver compounds ($AgNO_3$) on cell viability and apoptosis in keratinocytes and fibroblasts in primary cultures.

MATERIALS AND METHODS

Primary human keratinocyte culture

Specimens for culture were obtained from 3 circumcised patients. Foreskin was disinfected with 0.5% solution of chlorhexidine in 70% alcohol and then 1 cm^2 of skin was excised from the previously excised foreskin. All the experiments were complied with the Polish law and Local Ethical Committee recommendations. The number of permission issued by Local Ethical Committee in Collegium Medicum, Nicolaus Copernicus University in Bydgoszcz is 153/2004. The epidermis was incubated overnight in a 0.25% trypsin solution (Polfa, Poland) at 4°C. Then basal layer of the epidermis was scraped off. The scraped-off keratinocytes were centrifuged at $500 \times g$ for 5 min. Cells were cultured in medium contained DMEM/Ham's-12 at a 3:1 ratio, supplemented with 10% FBS. The medium

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was supplemented with insulin, transferin, triiodothyronine, hydrocortisone, EGF and antibiotics: penicillin and streptomycin (Sigma, Germany). The presence of keratinocytes in the culture was confirmed by an assessment of cell morphology (Fig. 1) and cytokeratin expression (Pancytokeratin Clone MMF-118; Dako, Denmark).

Primary human fibroblast culture

Skin fragments were cut into smaller fragments and left at the bottom of a 12-well culture plate (Corning, Costar) covered with a drop of medium. After fragment adhesion to the plate surface, the vessel was filled with DMEM supplemented with 10% FBS and antibiotics. The cultures were maintained until the growing area was covered (Fig. 2).

Fluorescence microscopy

Cells were examined under fluorescence microscopy to confirm that $AgNO_3$ induces apoptosis. To detect apoptotic and necrotic cells by fluorescence microscopy and flow cytometry Annexin V conjugated with fluoresceine isothiocyanate (Annexin V–FITC) and propidium iodide (PI) staining kit (Immunotech, Beckman-Coulter, USA) was used. Living cells were negative for Annexin V-FITC and PI. Early apoptotic cells were detected after binding with Annexin V-FITC. Secondary necrotic cells were stained positively both with Annexin V-FITC and PI. It is presumed that secondary necrotic cells died in apoptotic process. Necrotic cells were stained with PI.

Cells were incubated with $AgNO_3$ which was at 10 or 3 times lower concentrations, than is used in clinical practice. Two incubation periods were applied to mimicking period between change of dressings. Cells were incubated 3 h and 12 h with $AgNO_3$ in concentrations of 3 or 15×10^{-4} M/dm^3. Cells incubated with PBS (phosphate buffered saline, Polfa, Poland) were used as a positive control group. Then cells were rinsed with binding buffer (Immunotech, Beckman-Coulter, USA). Cultures were covered with 0.5 mL of binding buffer containing 0.05 mL of Annexin-V-FITC and 0.05 mL of PI. The samples were incubated for 30 min in darkness at 4°C. Immunofluorescence analysis was performed under inverted microscope (TS100, Nikon, Japan).

Flow cytometry

Annexin V conjugated with fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (IP) kit (Immunotech, Beckman-Coulter, USA) were used to detect apoptotic and necrotic cells. Approximately 10^5 keratinocytes or skin fibroblasts in 5 mL of medium was contained in 25 cm^2 flask (Greiner, Germany). $AgNO_3$ was added to cell culture after 24 h preincubation time. Cells were incubated 3 h and 12 h with $AgNO_3$ in concentrations of 3 or 15×10^{-4} M/dm^3. Incubation with PBS was used as a positive control group. Tested compounds were removed and then, cells were washed with binding buffer (Immunotech). Cells were suspended in 0.5 mL of binding buffer containing 0.05 mL of Annexin V-FITC and 0.05 mL of PI. The samples were then shaken for 5 s in an automatic stirrer (Coulter) and incubated 30 min in darkness at 4°C. Three separate measurements were performed. Determinations were performed using an EPICS XL flow cytometer (Coulter) with System 2 Software Version 1.0. Apoptotic cells were detected after binding with Annexin V-FITC. Necrotic cells were stained with PI. Secondary necrotic cells were

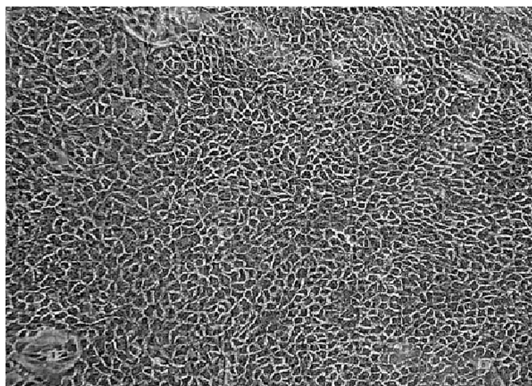

Figure 1. The monolayer of human keratinocytes in primary culture (inverted microscope, magnification 100×).

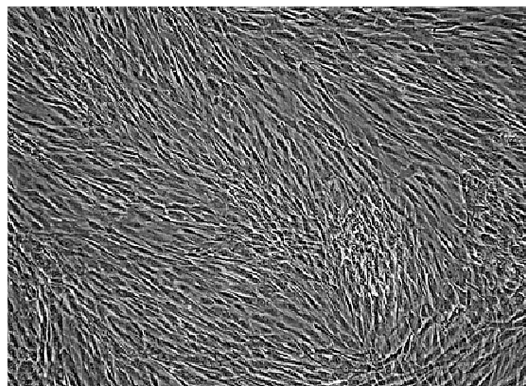

Figure 2. The monolayer of human fibroblasts in primary culture (inverted microscope, magnification 100×).

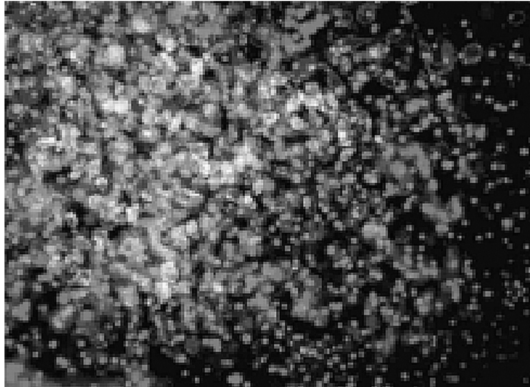

Figure 3. Human keratynocytes exposed to 15.0×10^{-4} M/dm³ $AgNO_3$ for 12 h (Annexin V-FITC and IP double staining, fluorescent inverted microscope, magnification 100×, merge).

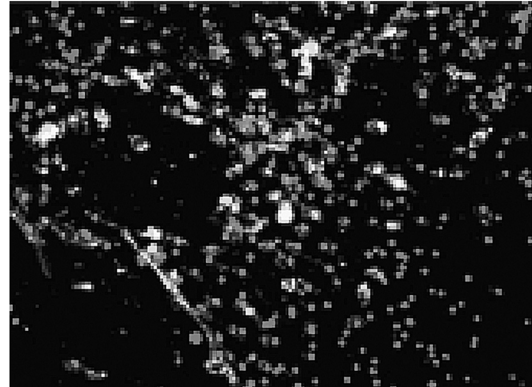

Figure 4. Human fibroblasts exposed to 15.0×10^{-4} M/dm³ $AgNO_3$ for 12 h (Annexin V-FITC and IP double staining, fluorescent inverted microscope, magnification 100×, merge).

stained by Annexin V-FITC and IP as well. It is presumed that secondary necrotic cells died in apoptotic process. Each value presented in the tables represents the mean of three measurements.

RESULTS

Three primary cultures of human keratinocytes and fibroblasts were established (Fig. 1 and 2). Apoptotic keratinocytes and fibroblasts were visible under fluorescence microscopy 3 or 12 h after addition of $AgNO_3$ (Fig. 3 and 4). The influence of $AgNO_3$ on the survival rate of primary cultured keratinocytes and fibroblasts was analyzed after 3 or 12 h of incubation in microscopy as a screening test. The viability of human fibroblasts cultured with PBS was greater than the viability of keratinocytes, reaching 84% for fibroblasts and 70% for keratinocytes, respectively (Fig. 5 and 6). Exposure of fibroblasts for 3 h to both concentrations of $AgNO_3$ caused a significant increase of apoptotic cell ratio (80%). The 45% decrease of apoptotic fibroblasts ratio was observed after 12 h (Fig. 6). The keratinocytes exposed to both concentrations of $AgNO_3$ responded with an increase of the apoptotic cell ratio. This effect was observed after 3 and 12 h (Fig. 5). The percentage of keratinocytes which showed signs of necrosis after 12 h of incubation with $AgNO_3$ was similar to control (Fig. 5).

DISCUSSION

We have previously proved that $AgNO_3$ is toxic for 3T3 mouse fibroblasts (4). It seems that the use of primary cultures rather than immortalized cell lines is relevant for the interpretation of results and the assessment of their real value (5). In cytotoxicity studies primary cultures reflect better *in vivo* conditions, when compared to cell lines (6). Heterogeneity of cells harvested from individuals and then used for testing is an unquestionable advantage of this method (7). Low success rate of culture establishment and lower cell viability during passages are due to apoptosis of some cells related to detachment (anoikis) and are limiting factors of *in vitro* studies with primary cell cultures. Three primary cultures of human fibroblasts and keratinocytes were used in this study. The control fibroblasts incubated with PBS showed greater viability than the control keratinocytes. The higher viability of fibroblasts may be related to stimulatory effect of PDGF from bovine serum (8-10). The number of apoptotic keratinocytes in control group increased from 20.2% to 26.4% after 3 and 12 h, respectively. No change was recorded in the number of apoptotic cells in the fibroblast control group. Keratinocytes were more sensitive to trypsinization and detachment. Epithelial cells undergo anoikis more likely than fibroblasts (11). The fact that keratinocytes have lower viability in comparison to fibroblasts may lead to delayed epithelialization and impaired wound healing in burned patient (12). Intensive fibroblast growth and paracrine influence can facilitate tissue granulation and scar formation (9, 10, 13). Reverse phenomenon was also presented. Intensively dividing fibroblasts enhanced wound epithelialization (14). Apoptotic fibroblasts and keratinocytes were observed after 3 h

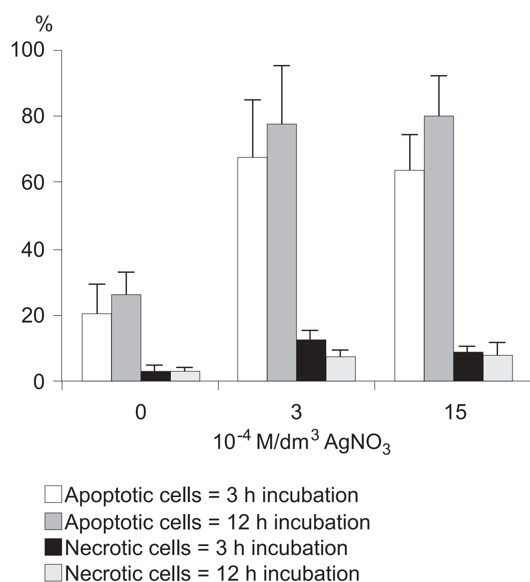

Figure 5. The $AgNO_3$ influence on human keratinocytes in primary cultures.

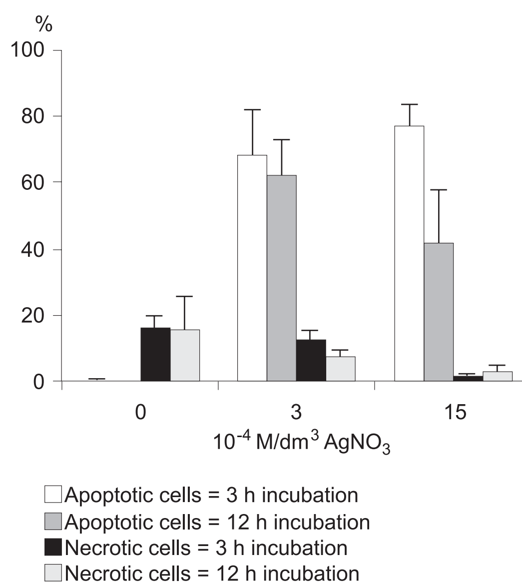

Figure 6. The $AgNO_3$ influence on human fibroblasts in primary cultures.

at both investigated $AgNO_3$ concentrations. After 12 h the percentage of apoptotic cells was greater in the keratinocyte population when compared to the fibroblast population. Keratinocytes were more susceptible to $AgNO_3$ than fibroblasts during 12 h experiment. The incubation time influenced cell viability. 0.5% (3.1×10^{-3} M/dm^3) $AgNO_3$ solution used in burned patients is ten times more concentrated than the solutions used in our experiment. The wound environment always contains a certain amount of fluid. Within the wound, $AgNO_3$ solution reached concentrations comparable to those investigated. Szmytkowska has noted that the use of silver compounds in management of burned patients delayed outcome and led to hypertrophic scar (2). If the thesis that the healing and scar formation in burns is related to the balance between the cell populations that form the epithelium and the granulation tissue is considered, then the results presented in this paper show that $AgNO_3$ decreases the viability of both cell populations and disrupts the balance between the number of keratinocytes and fibroblasts (15). Keratinocytes secrete factors promoting fibroblast differentiation, thus inhibits fibroblast divisions (15). Clinical observations together with results presented here support conclusion that the presence of hypertrophic scars is related to inhibited epithelialization. $AgNO_3$ solutions may further exacerbate this unfavorable process. From a clinical point of view, it seems important to identify conditions, under which the quantitative balance of epidermal cells would be shifted in favor of the keratinocytes (17). The decreased viability and increased sensitivity of keratinocytes to silver compounds in the 3 and 12 h test may provide an explanation for the disrupted epithelialization and hypertrophic scar formation observed in burned patients treated with compounds containing silver ions (18). Silver based products should be used with caution or replaced with antibiotics if burned wound was grafted with cultured cells (19).

CONCLUSIONS

Human fibroblasts and keratinocytes underwent apoptosis when had been exposed to bactericidal concentrations of silver ions. The ointments containing silver nitrates should be used with caution especially in conditions where the epithelial layer has been destroyed. Silver nitrate can negatively influence on wound healing.

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