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The effect of hyaluronan combined with KI_3 complex (Hyiodine wound dressing) on keratinocytes and immune cells

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Abstract Hyiodine (high molecular weight hyaluronan combined with KI_3 complex) is a new non-adhesive wound dressing which significantly improves the healing process. The aim of the study was to investigate the effects of Hyoidine on functional properties of isolated human keratinocytes and leukocytes, and on those of U937 and HL60 cell lines. While KI_3 complex inhibited the viability and proliferation of the cells tested, Hyiodine did not have any significant effect. The expression of CD11b, CD62L and CD69 on PMNL, monocytes and lymphocytes, as well as the oxidative burst of blood neutrophils, were not changed. On the contrary, Hyiodine inhibited the PMA-activated oxidative burst and significantly increased the production of IL-6 and TNF-α by lymphocytes. It was concluded that hyaluronan content of Hyiodine reduces the toxic effect of KI_3 complex on cells and speeds up the wound healing process by increasing the production of inflammatory cytokines.

Abbreviations: CL, chemiluminescence; HA, hyaluronan; PMNL, polymorphonuclear leukocytes; ROS, reactive oxygen species

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Introduction

Effective wound healing requires a highly organized series of events that comprises inflammation, re-epithelialization, keratinocyte proliferation, matrix deposition, angiogenesis, and wound contraction [1]. Together, these processes result in the restoration of tissue integrity and functional healing. The wound healing process is influenced by the immune status of the host, and by conditions such as advanced age, corticosteroid administration, or systemic diseases such as diabetes mellitus. Of the human population with diabetes, 15% will develop a foot ulcer at some point in their lives and a purported 20% of diabetes-related foot ulcers ultimately will result in lower limb amputation with high mortality in the subsequent five years. Worldwide, the number of amputations continues to increase and appropriate treatment of ulcers and diabetic wounds still constitutes a significant challenge for current medicine [2].

Many of the factors required for wound healing are generated during inflammation. These include growth factors, cytokines, and eicosanoids, which promote migration of the inflammatory cells, fibroblasts, and endothelial cells into the wound site. A crucial role, in the defence against invading pathogens, play phagocytes mainly neutrophils and monocytes migrating from blood circulation and tissue macrophages. Effective neutrophil antimicrobial action depends on the generation of oxygen-derived free radicals formed during the respiratory burst of phagocyte and on the release of proteolytic enzymes. Interestingly, in individuals with diabetes, impaired healing and infection of diabetic wounds are connected with alterations in neutrophil function (deficiencies in neutrophil chemotaxis, phagocytosis, respiratory burst activity, and intracellular killing) in individuals with diabetes [3].

Bacteria colonize essentially all chronic wounds, and result in an infection when bacterial proliferation outstrips the ability of the host defenses to contain it. Bacteria stop a healing process and can spread into surrounding unwounded tissue as well. Topical antimicrobial agents are used clinically on a variety of infected wounds and the object of their use is the control of microbial proliferation. Iodine-containing antibacterial preparations (povidone-iodine ointment and cadexomer iodine gel) belong to the commonly used agents and provide a broad antibacterial and antifungal spectrum. However, in evaluating of the cytotoxicity of common topical antimicrobial agents, iodine solutions were shown to be significantly toxic *in vitro* to both fibroblasts and keratinocytes which could significantly impaired wound healing [4]. Therefore, combining iodine with agents stimulating wound healing and decreasing adverse effects connected with the use of a topical iodine application, including allergic reactions, and iodine toxicity would significantly improve the use of iodine as a topical antimicrobial agent.

Hyaluronan (HA) is a major extracellular matrix molecule in the vital cell layers of skin epidermis, plays important role in wound healing [5]. HA is a high-molecular-weight polymer synthesized as disacharide repeats of D-glucuronic acid and N-acetyl-D-glucosamine which due to its polyanionic and hydrophilic properties provides structure and viscosity to the extracellular matrix [6]. In normal epidermis, HA traps large amounts of water, contributing to tissue hydration, viscosity but also edema that followed tissue injury. The ability of hyaluronan to coli and self-associate may also contribute to tissue elasticity. HA can support proliferation and migration of keratinocytes during challenges like wounding and inflammation. HA promotes the formation both of early granulation tissue and of the smooth wound surface considered to be a proper wound bed for autografting [7].

In the granulation phase, HA plays a role in macrophage and neutrophil activation, in the formation of new blood vessels, and in the deposition of various extracellular matrix proteins [8]. In terms of re-epithelialisation, it is well documented that HA aids keratinocyte proliferation and migration, and has also been implicated in fetal scarless wound healing. The majority of cells in the body have the capability to synthesize HA during their cell cycle, implicating its function in several fundamental biological processes such as embryogenesis, wound healing, tissue regeneration, leukocyte-endothelial cell interaction and tumour metastasis. HA oligosacharides bind to the CD44 receptor on macrophages, inducing the expression of several inflammatory genes such as TNFα and IL1β. Furthermore, HA facilitates the primary adhesion of cytokine-activated lymphocytes expressing the hyaluronan-binding variants of CD44 under laminar and static flow conditions [9].

There is evidence in the biomedical literature proving an involvement of HA in the process of wound repair and the beneficial role of exogenously added HA in the acceleration of the wound healing. The esterified form of HA (Hyaff-11) has been used as a scaffold for skin, cartilage and as a vascular scaffold [10]. Due to its unique properties, HA is used as a scaffold for healing diabetic wound where the normal healing process of ulcers and diabetic wounds is often impaired [11].

To further improve the beneficial properties of HA, the new non-adhesive wound dressing Hyiodine, where the effects of high molecular weight HA is combined with the action of KI_3 complex, was developed especially for the medical treatment of non-healing wounds such as leg ulcers and diabetic wounds. Data from our clinical study showed a highly significant improvement of healing process after Hyiodine treatment of patients with non-healing wounds, infection, diabetic leg ulcers and large wounds. In this study, we followed the effects of Hyoidine on proliferation and functional properties of keratinocytes and cells of the immune system and compared them with the effects of KI_3 complex.

Materials and methods

The complex of hyaluronan and KI_3 (Hyiodine, Contipro C, Czech Republic) is antiadhesive wound healing dressing for non-healing wound, leg ulcers and diabetic wound. Hyiodine is sterile and it is used as a scaffold for covering, hydrating and cleaning deep wound and skin defect. It has strong affinity for water and combines wound healing and angiogenic properties of hyaluronan together with antibacterial properties of complex KI_3.

Hyiodine consists of 1.5% hyaluronan (molecular weight 1.5 MDa) and KI_3 complex (0.1% I_2 and 0.15% KI) in water for injection (patent No: WO 03/059404). Two concentrations of Hyiodine (0.5, 1 mg/ml) were tested against one concentration of KI_3 complex (equivalent to the amont found in 1 mg/ml Hyiodine)

Primary normal human epidermal keratinocytes (NHEKs) were isolated from skin removed during cosmetic plastic surgery. The samples were obtained with informed consent and institutional approval. Keratinocyte cultures were prepared according to the method of Reinwald and Green (1975). The skin was minced and trypsinized (0.25% trypsin + 0.02% EDTA, Biomedia, Brno, Czech Republic) at 37°C for 30 min and keratinocytes were grown in 75 cm^2 culture flasks with mitomycin–treated 3T3 fibroblasts. Keratinocytes were grown in complete medium containing DMEM (Biomedia, Brno, Czech Republic), HAM-F-12 (Sigma-Aldrich, St. Louis, MO, USA), fetal calf serum (10%,

Gibco, Paisley, Scotland), hydrocortisone, adenine, Cholera Toxin, EGF, insulin, amphotericin B, penicilin, streptomycin, apo-transferin, 3,3′,5-triiodo-L-thyronin (Sigma-Aldrich, St. Louis, MO, USA). When keratinocytes reached confluency, the cells were washed twice in PBS and cultures were passage in secondary cultures. Cells were plated at $1 * 10^4/cm^2$ mitomycin-treated 3T3 fibroblasts. The medium was changed every two days. After three passages cells were seeded on 6-well plates (7 cm^2) in the concentration of $0.14 * 10^6$ cell per well for ELISA and on 96-well tissue culture plates in the concentration of $5.6 * 10^3$ cell (0.28 cm^2) per well for cell proliferation assay. The cells were growing for 24 h and then Hyiodine and KI_3 complex were added and incubated with cells for 24 or 48 h at 37°C and 7.5% CO_2. Untreated cells were used as a control. After incubation periods, the culture medium was collected and stored at −20°C until the ELISA assay was performed. RNA was isolated and stored at −20°C in DEPC water.

Blood leukocytes

Heparinized (50 IU/ml) blood samples were obtained from healthy volunteers with given informed consent. The number of leukocytes in the blood and their relative differentiation counts were determined using Coulter counter STKS (Coulter, England), and stained blood smears, respectively. Leukocytes were isolated as described previously [12]. Blood was layered in a ratio of 1:1 over the separation mixture. The separation mixture consisted of 4% Dextran-T500 (Amersham Biosciences AB, Sweden) in saline and 60% telebrix N 300 (Leciva, Dolni Mecholupy, Czech Republic) in saline in a ratio of 3.7:1 and its final density was 1.08 g/cm^3. Erythrocytes were removed after 1 h of sedimentation at room temperature and leukocytes with plasma were obtained. Then, one-third of leukocytes were washed in RPMI-1640 (200 g, 5 min) and the rest of leukocytes were added to Ficoll-Pague (Amersham Biosciences AB, Sweden) for separation of lymphocytes and polymorphonuclear leukocytes (PMNL). Then the cells were resuspended to reach a final density $1 * 10^6$ cells/ml in RPMI-1640 supplemented with 10% heat-inactivated human AB serum. Whole blood samples were incubated with Hyiodine and KI_3 complex at 37°C for 1 h prior to determining their oxidative burst by chemiluminescence (CL) and hte expression of surface antigens on PMNL by flow cytometry. Isolated leukocytes were incubated with Hyoidine and complex KI_3 in RPMI-1640 medium supplemented with 10% of heat-inactivated human AB serum and gentamycin sulphate at 37°C for 18 h. Hanks balanced salt solution, pH 7.4 was used instead of polysaccharides as a control. Concentrations of selected cytokines were determined in supernatant at the end of incubation.

U937 and HL60 cell lines

U937 (human monocyte-like cells) originate from diffuse histiocytic lymphoma. They have aneuploid karyotype, display many monocytic characteristics and serve as an in vitro model for monocyte/macrophage differentiation. HL60 (human neutrophil-like cells) are of lymphoma origin. Both cell lines were grown in RPMI-1640 supplemented with heat-inactivated foetal calf serum (10%) (Gibco, Paisley, Scotland), glutamin 250 g/ml (Gibco, Paisley, Scotland), gentamycin (Gibco, Paisley, Scotland), PYNA 0.1 mg/ml (Lachema, Neratovice, Czech Republic), HEPES 1 M (Gibco, Paisley, Scotland), and 2-mercaptoethanol 4.456 μg/ml (Sigma-Aldrich, St. Louis, MO, USA). The medium was changed every two days and cells were seeded with a final concentration $3 * 10^5$ per well in 6-well plates (surface of well 7 cm^2) and $1.2 * 10^4$ per 96-well plates (surface of well 0.28 cm^2). Hyiodine and complex KI_3 were added and incubated with cells for 24 or 48 h at 37°C and 5% CO_2. Untreated cells were used as controls. After incubation periods, the culture medium was collected and stored at −20°C until the ELISA assay was performed.

Expression of genes for keratins

Total RNA was extracted from NHEKs. Total RNA was reverse transcribed in 22 μl reaction mixture containing Random Primer 3 μg (1 μl, Gibco, Paisley, Scotland), DEPC water 5.1 μl, 1st Strand buffer (Gibco, Paisley, Scotland), DTT 0.1 M (3 μl, Gibco, Paisley, Scotland), Rnase OUT^{tm} Ribonuclease Inhibitor Recombinant 5000 U (1 μl, Gibco, Paisley, Scotland), dNTP 2.4 μl, BSA 3.0 μl, and Superscript^{tm}II RT 10,000 U (1.5 μl, Gibco, Paisley, Scotland)

RT-PCR was performed in 20 μl of RT-PCR mix containing DEPC water (13.4 μl), PCR Buffer 10 × (2.5 μl, Gibco, Paisley, Scotland), $MgCl_2$ 50 mM (1.5 μl, Gibco, Paisley, Scotland), BSA 1 mg/ml (1 μl, Gibco, Paisley, Scotland), oligonucleotides 10 μM (0.5 μl, Gibco, Paisley, Scotland), dNTP 0.5 μl, and Tag DNA Polymerase Recombinant 500 U (0.1 μl, Gibco, Paisley, Scotland).

Amplification of human K1, K5, K10 and K14 was performed using oligonucleotides as described [13] K5: 5′-CAGCGTCAAATTTGZCTCCAC-3′ and 5′-TTGGTCTAGACTACTCTCCAG-3′; K14: 5′-CGCCAAATCCGCACCAAGGTC-3′ and 5′-GAAGCAGGGTCCAGCTGTGAA-3′; K1: 5′-CACTTATTCCGGAGTAACCAG-3′ and 5′-GAATAGGATGAGCTAGTGTAA-3′; K10: 5′-GAGTCTTCATCTAAGGGACCA-3′ and 5′-AATGGTCTGTGTGAAGGGAGA-3′. We used Thermal cycler (Peltier,

France) and gel documentation system (Syngene, France).

Cell viability

The viability and metabolic activity of cells, which were previously incubated with different concentrations of Hyiodine and KI_3 complex, was performed using the XTT cell proliferation kit (Roche, Mannheim, Germany). The principle of this colorimetric test is based on mitochondrial cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye. This conversion occurs only in metabolic intact cells. Cells were seeded in 96-well microplates for 24, 48, 76 or 92 hrs. The absorbance was measured after 2 h at 500 nm against a reference wavelength of 650 nm, using a microtiter plate reader (Molecular Devices UK, VERSA Max software UK).

Cytokine determination

The determination of cytokines in supernatant was performed by sandwich ELISA according to the manufacturer's protocol for IL-6 Modul Set, IL-8 Modul Set, IL-12 Modul Set and TNFα Modul Set (BenderMedSystems, Vienna, Austria). The absorbance was measured at 450 nm against a reference wavelength of 620 nm, using a microtiter plate reader (Molecular Devices UK, VERSA Max software UK).

Oxidative burst of blood phagocytes

Luminol-enhanced CL of whole blood phagocytes was measured using a microplate luminometer LM-01T (Immunotech, Czech Republic) as described previously [14]. The principle of the method is based on luminol interaction with the phagocyte-derived free radicals, which results in large measurable amounts of light. Briefly: The reaction mixture consisted of 10 μl whole blood, 1 mM luminol (stock solution of 10 mM luminol in 0.2 M borate buffer) and the activator (PMA–0.5 μM). Stock solution of 10 mM activator was prepared in dimethylsulfoxide. The final concentration of dimethylsulfoxide did not exceed 0.1%, which was proved not to affect CL reaction. The total reaction volume of 200 μl was adjusted with Hanks balanced salt solution. The assays were run in duplicates. Spontaneous CL measurements in samples containing 10 μl of whole blood and other substances except the activator were included in each assay. The CL emission expressed as relative light units (RLU) was recorded continuously for 90 min at 37°C. The integral value of the CL reaction, which represents the total reactive oxygen species (ROS) production by blood phagocytes, was corrected to the number of PMNL.

Determination of the expression of cell surface molecules

The measurements were performed according to the manufacturer's protocol (Caltag Laboratories, USA) with minor modifications as described previously [15]. Briefly, 100 μl of blood were incubated with anti-CD11b and anti-CD62L monoclonal antibodies at room temperature for 15 min. Isolated lymphocytes (10^5) in 100 μl of PBS with 1% bovine serum albumine were incubated with anti-CD69 monoclonal antibody at 4°C for 30 min. Samples were incubated with FITC-conjugated or PE-conjugated murine immunoglobulins of the same isotype were used as the negative controls. Then the blood samples were fixed by Cal-lyse and the red blood cells were lysed by distilled water. Isolated lymphocytes were washed once with ice cold PBS. The remaining cells were resuspended in PBS, placed on ice and analysed within 2 h. At least ten thousand PMNL, one thousand monocytes or ten thousand lymphocytes were collected based on a selection according to their typical scattering characteristics and expression of antigens were analysed by flow cytometer FACS Calibur (Becton Dickinson, USA). The geometric mean of relative fluorescence units (RFU) was quantified and corrected to the background fluorescence of the isotype control in the case of CD11b and CD62L determination.

Statistical analysis

The Wilcoxon non-parametric tests were applied for the analysis of results from flow cytometry and oxidative burst results using software Statistics for Windows 5.0 (Statsoft, USA).

Multifactorial ANOVA referring 2 independent factors (time, sample) was used for the analysis of results from ELISA, XTT and RT-PCR results (software STATGRAPHICS Plus 5.1 and QC-Expert 2.5PRO). The power transformation of the data was used in some cases.

Results and discussion

The new non-adhesive wound dressing Hyiodine (high molecular weight hyaluronan combined with KI_3 complex) significantly improves the healing process of non-healing wounds, diabetic leg ulcers and large wounds [16]. However, effect of Hyiodine on proliferation, viability and activation of human cells *in vitro* is not known. In the present study, keratinocytes isolated from human skin together with human leukocytes isolated from peripheral blood or derived from two stable cell lines were used to test the effect of Hyiodine on their proliferation, viability, activation or differentiation based on determination of production of cytokines,

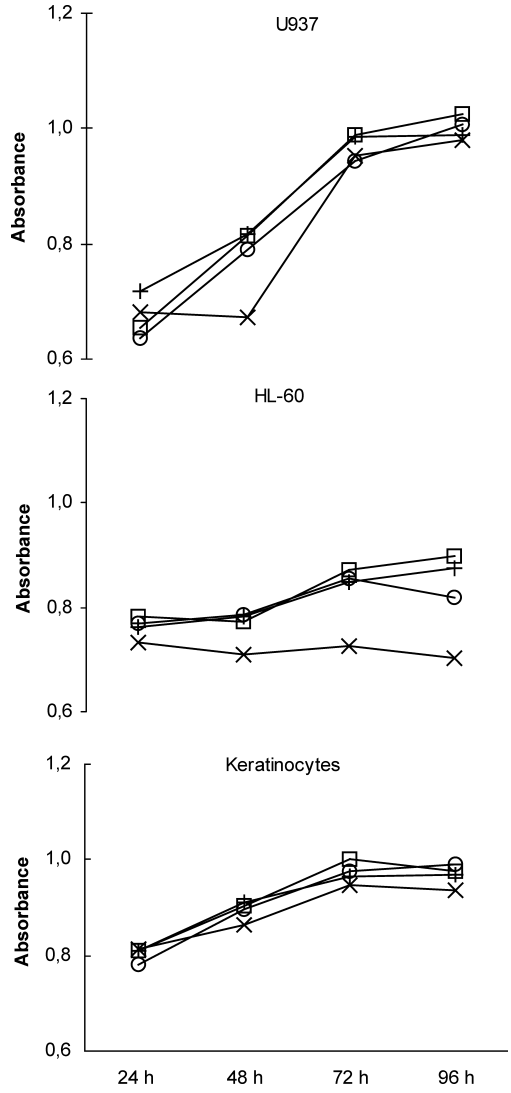

Fig. 1 Effect of Hyiodine and KI_3 complex on cell viability. Cell lines U937, HL-60 and primary normal human epidermal keratinocytes were divided into 4 groups: controls (□), cells co-incubated with 0.5 mg/ml of Hyiodine (+), with 1.0 mg/ml of Hyiodine (○) or with KI_3 complex (×). Cell proliferation was measured colorimetrically using a XTT cell proliferation kit. Data are expressed as means and lower and upper limits of 95 percent LSD interval.

expression of surface receptors, production of reactive oxygen species during oxidative burst of phagocytes, or expression of keratins.

First of all we tested the effect of Hyiodine and complex KI_3 on the viability of keratinocytes, HL60 and U937 by XTT test. While KI_3 complex slightly inhibited proliferation of all cells tested (the effect being statistically significant in HL-60 cells), both concentrations of Hyiodine did not have a significant effect on the viability and proliferation of these cells (Fig. 1). These findings prove the combination of HA with complex KI_3 in Hyiodine wound dressing does not have a negative effect on cell growth. None of the tested concentrations of Hyiodine or KI_3 changed the expression of surface receptors CD11b, CD62L and CD69 on PMNL, monocytes or lymphocytes after 3 h incubation of Hyiodine and complex KI_3 with whole blood (data not shown). In our previous studies, HA did not have an effect on blood leukocytes activation (unpublished data). These findings were consistent with other studies documenting the lack of pro-inflammatory effect of high m. w. HA on leukocytes.

Neither Hyiodine nor KI_3 complex did change the spontaneous production of ROS by intact phagocytes (Fig. 2). On the other hand, PMA-induced ROS production was significantly decreased by both concentrations of Hyiodine. KI_3 complex inhibited PMA-induced ROS production by phagocytes similarly as Hyiodine. Moseley *et al.* [17] observed that high molecular weight hyaluronan in concentrations of up to 1 mg/ml did not influence the ROS production by phagocytes. The results of these authors together with our results suggest that the presence of KI_3 complex in Hyiodine could be responsible for the inhibition of PMA-activated oxidative burst of phagocytes. It could be important since phagocyte derived ROS can damage cells and tissue during wound healing.

As mentioned above, production of pro-inflammatory mediators at the site of the wound was proved to significantly increase the wound healing process. IL-6 and TNF-α are important factors for inflammation, the first stage of wound healing [18–20]. They stimulate keratinocytes and fibroblasts proliferation and migration, and regulate immune response [21]. IL-8 acts as a chemoatractant for neutrophils [22] and is capable of stimulating migration and proliferation of keratinocytes. IL-12 (cytotoxic lymphocyte maturation factor) stimulates the differentiation of Th1 from progenitor cells. It is inductor of INFγ and inhibitor of angiogenesis [23]. Therefore, the hypothesis that Hyiodine can speed up the process of wound healing through modulation of inflammatory cytokines (IL-6, IL-8, IL-12 and TNF-α) release was tested.

Hyiodine significantly increased the production of IL-6 and TNF-α by lymphocytes after 24 h but in comparison with control (Table 1), while there was no significant difference between Hyiodine concentrations 0.5 and 1 mg/ml. Complex KI_3 slightly increased the production of studied cytokines. The only statistically significant difference between Hyiodine and complex KI_3 was found in the case of TNF-α for 1 mg/ml Hyiodine concentration. The productions of IL-8 and IL-12 were increased non- significantly by both concentration of Hyoidine or KI_3 complex.

The production of all cytokines studied by HL-60 cells was around detection limit and neither Hyiodine nor KI_3 complex induced any changes after 24 h or after 48 h (data not shown). The production of all cytokines by keratinocytes

Table 1 The production of cytokines by lymfocytes after 24 h of co-incubation with Hyiodine or KI_3

	Control	Hyiodine 0.5 mg/ml	Hyiodine 1.0 mg/ml	KI_3 complex
IL-6	2.02^{A} (2.25; 1.80)	2.54^{B} (2.77; 2.32)	2.67^{B} (2.90; 2.44)	$2.32^{A,B}$ (2.55; 2.09)
IL-8	1325^{A} (1631; 1019)	1589^{A} (1895; 1283)	1858^{A} (2164; 1552)	1663^{A} (1969; 1357)
IL-12	232^{A} (347; 116)	287^{A} (403; 172)	298^{A} (414; 183)	313^{A} (429; 198)
TNF-alpha	4.27^{A} (4.90; 3.65)	$5.56^{B,C}$ (6.19; 4.93)	5.95^{C} (6.58; 5.32)	$4.54^{A,B}$ (5.17; 3.92)

Data are expressed as means calculated from original values (pg/ml) normalized by power transformation. Lower and upper limits of 95 percent LSD intervals are shown in parentheses. There are no statistical significant differences among values marked by the same letter.

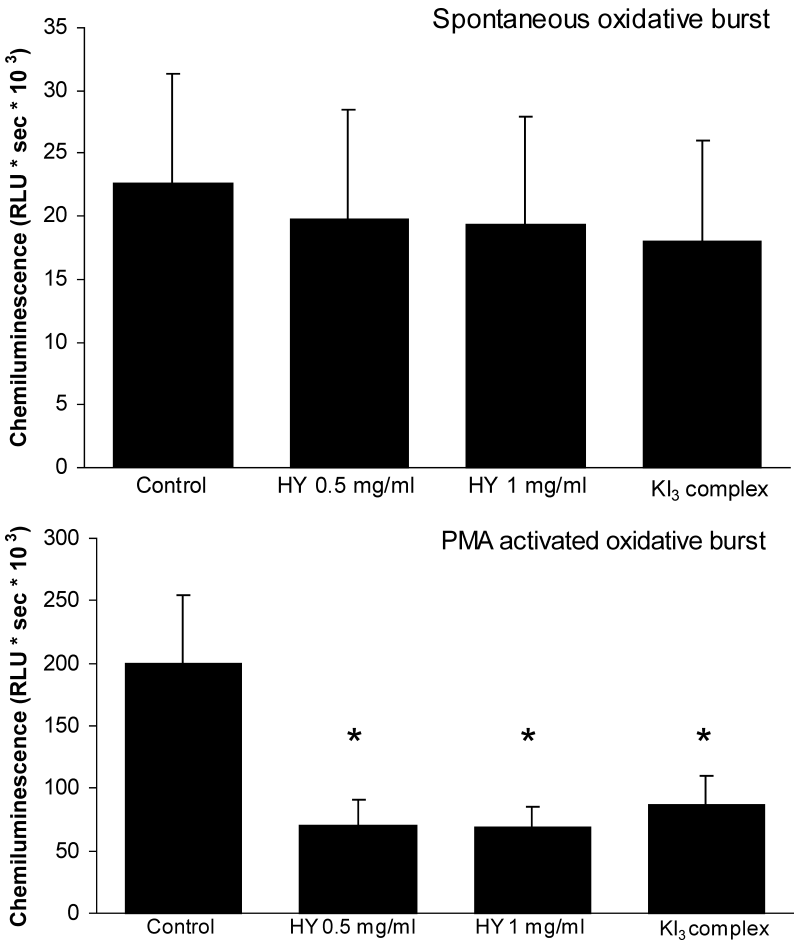

Fig. 2 Influence of Hyiodine and KI_3 complex on the spontaneous and PMA-activated oxidative burst of blood phagocytes. The oxidative burst of phagocytes was analysed chemiluminometrically. Light emission expressed as relative light units (RLU) was recorded continuously for 90 min at 37°C and corrected to the number of polymorphonuclear cells. Data are expressed as means ±S.D. Asterisks mark the statistically significant differences between given values and controls ($p \leq 0.05$).

and U937 was decreased after 24 h but increased after 48 h was (data not shown).

In healthy epidermis, keratinocytes differentiate from the basal layer through squamous, granular, and cornified layer. The differentiation between basal and differentiating keratinocytes can be performed by the analysis of expressions of genes specific for structural fibrous proteins–keratins. Based on amino acid sequence, charge, size, immunological properties and homologies with wool keratins, the human keratins are organized into two families, the type I (K10–K20, acidic) and type II (K1–K9, basic-neutral) [24]. We studied the influence of Hyiodine on expression of K5 and K14 keratins expressed in the basal layer, as well as on expression of K1 and K10 keratins expressed in the differentiating cells [25–27]. After 24 h incubation with Hyiodine or KI_3 complex the expression of K5 gene was significantly decreased while expression of K14 gene was significantly increased in comparison with untreated control. On contrary, none of substances tested changed the expression of these keratins after 48 h incubation. Genes for K1 and K10 were expressed neither in control cells nor in cells co-incubated with Hyiodine or KI_3 complex (Fig. 3).

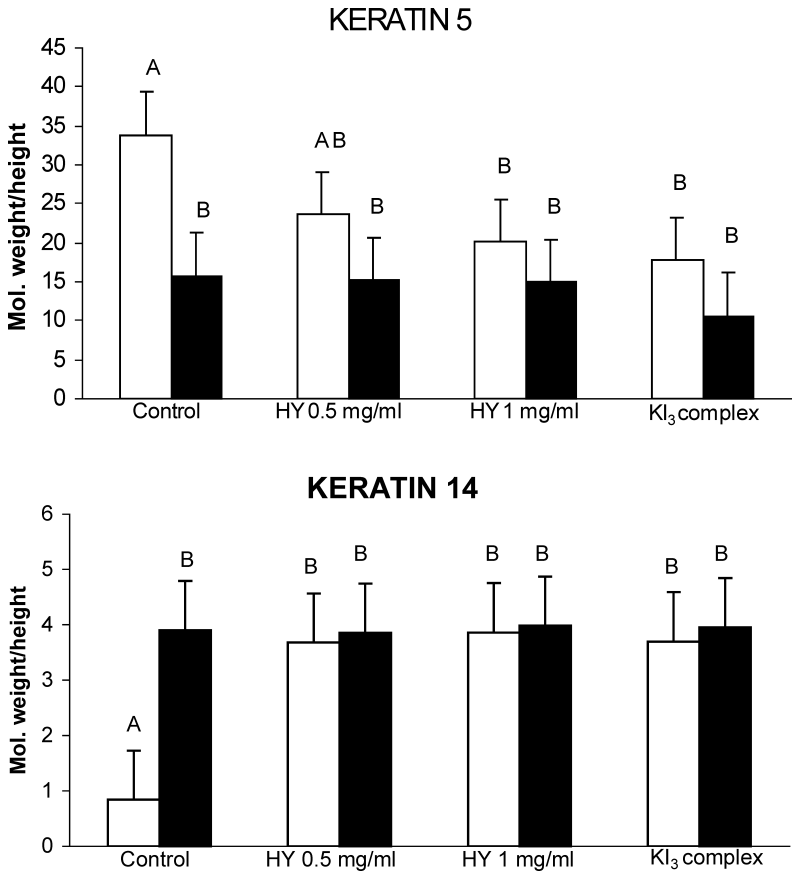

Fig. 3 Effect of Hyiodine and KI_3 complex on Keratin 5 and Keratin 14 genes expression in keratinocytes. RNA was isolated after 24 h (empty bars) and 48 h (full bars) of co-incubation with substances tested and stored at −20°C in DEPC water until RT-PCR analysis. Data are expressed as means of peak heights (obtained using gel documentation system) and lower and upper limits of 95 percent LSD interval. There are no statistically significant differences among the values in bars marked by the same letters.

It proves that all keratinocytes after 24 and 48 h of incubation belong to cells typical for basal layer. Observed phenomenon of decreased and increased expression of genes for K5 and K14, respectively, cannot be clearly explained based on existing literature and it remains to be elucidated further.

Conclusion

The combination of HA with KI_3 complex in the Hyiodine does not have toxic effect on cells. Further, Hyiodine significantly modulated production of pro-inflammatory cytokines. Both of these effects observed *in vitro* can contribute to significantly improved process of wound healing observed in our previous clinical study.

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