INFORMATION CAPACITY OF THE NF-κB AND AP-1 SIGNALING ACTIVATION SENSORS IN *IN VITRO* ASSESSMENT OF DERMATOTOXIC EFFECTS

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Toxicity testing, including testing for skin toxicity, is essential for certification of novel pharmaceutical, chemical, and skincare products. The *in vitro* assessment models are considered to be the most promising; a number of such tests have been introduced into practice of approval testing. The new possibilities of detecting the early cellular response to damage can be provided by the cell-based sensors built upon visual quantification of the changes in activity of the signaling pathways involved in realization of such response. NF-κB and AP-1 represent two important protein transcription factors, the increase in activity of which in the cell is associated with damage, inflammation or redox balance alteration. The study was aimed to develop the cell-based sensors built upon the HaCaT immortalized human keratinocyte cell line that express green fluorescent protein (GFP) when the NF-κB (HaCaT/NF-κB) or AP-1 (HaCaT/AP-1) signaling pathway is activated, as well as to assess their information capacity when recording the dose-dependent response to the exposure to inducers of appropriate signaling pathways. The findings showed that the HaCaT/NF-κB cell fluorescence levels changed by 6.05 ± 0.51 and 5.53 ± 0.52 times upon exposure to TNFα or LPS (at a concentration of 0–80 ng/mL) in a dose dependent manner. The HaCaT/AP-1 biosensor also responded to the exposure to Cd $(NO_3)_2$ (at a concentration of 0–40 μM) and ultraviolet A (UVA) (0–40 J/cm^2), however, it enabled qualitative, but not quantitative detection. The censor cell fluorescence increased by 1.51 ± 0.24 and 1.66 ± 0.43 times, respectively. The cell-based sensors developed can be used to assess cytotoxic effects of the test substances on the human skin cells *in vitro* and study the cytotoxicity mechanisms.

Keywords: keratinocytes, biosensor, skin toxicity

Funding: the work was performed within the framework of the Program for Basic Research in the Russian Federation for a long-term period (2021–2030) (№ 122022800481-0).

Author contribution: Tolstova TV — experimental procedure, data acquisition, analysis, and interpretation, manuscript writing, illustrations; Pureczcky VK — experimental procedure, illustrations; Kozhin PM — literature review, manuscript writing, illustrations; Luzgina NG — study concept and design, manuscript writing; Rusanov AL — study planning, manuscript writing, overall guidance for the study.

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Received: 11.12.2023 **Accepted:** 16.02.2024 **Published online:** 27.02.2024

DOI: 10.24075/brsmu.2024.007

ИНФОРМАТИВНОСТЬ СЕНСОРОВ АКТИВАЦИИ СИГНАЛЬНЫХ ПУТЕЙ NF-κB И AP-1 ПРИ ОЦЕНКЕ ДЕРМАТОТОКСИЧЕСКИХ ЭФФЕКТОВ *IN VITRO*

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Для сертификации новых фармацевтических, химических или косметических продуктов необходимо тестирование на токсичность, в том числе дерматотоксичность. Модели *in vitro* исследований считают наиболее перспективными, и ряд таких тестов внедрен в практику сертификационных испытаний. Новые возможности для регистрации раннего ответа клеток на повреждение могут предоставить клеточные сенсоры, основанные на визуальной количественной регистрации изменений активности сигнальных путей, задействованных в реализации такого ответа. NF-κB и AP-1 — два важных фактора транскрипции белков, активность которых возрастает в клетке при повреждении, воспалении и изменении редокс-баланса. Целью исследования было разработать клеточные сенсоры на основе иммортализованных кератиноцитов человека линии HaCaT, которые экспрессируют зеленый флуоресцентный белок (GFP) при активации сигнальных путей NF-κB (HaCaT/NF-κB) или AP-1 (HaCaT/AP-1), и изучить их информативность при регистрации дозозависимого ответа на воздействие индукторов соответствующих сигнальных путей. Результаты показали, что уровень флуоресценции клеток HaCaT/NF-κB дозозависимо изменялся в 6,05 ± 0,51 и 5,53 ± 0,52 раз при воздействии TNFα или LPS (в концентрациях от 0 до 80 нг/мл). Биосенсор HaCaT/AP-1 также реагировал на воздействие Cd $(NO_3)_2$ (в концентрациях от 0 до 40 мкМ) и ультрафиолетового излучения типа А (УФ-А) (от 0 до 40 Дж/см^2), однако позволял регистрировать его качественно, но не количественно. Флуоресценция клеток сенсора возрастала в 1,51 ± 0,24 и 1,66 ± 0,43 раз соответственно. Разработанные клеточные сенсоры могут быть использованы для оценки цитотоксического действия тестируемых веществ на клетки кожи человека *in vitro* и изучения механизмов цитотоксичности.

Ключевые слова: кератиноциты, биосенсор, дерматотоксичность

Финансирование: работа выполнена в рамках Программы фундаментальных научных исследований в Российской Федерации на долгосрочный период (2021–2030 годы) (№ 122022800481-0).

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Статья получена: 11.12.2023 **Статья принята к печати:** 16.02.2024 **Опубликована онлайн:** 27.02.2024

DOI: 10.24075/vrgmu.2024.007

The risk of skin injury or irritation represents one of the restrictions for implementation of novel pharmaceutical substances, chemical compounds, and skincare products. In this regard, the approval safety testing of such products involves mandatory skin toxicity testing.

There is a number of routine *in vivo* tests approved by the Organization for Economic Cooperation and Development (OECD) and regulated by GOST of the RF. In particular, the OECD protocol № 429 based on the murine local lymph node assay [1], protocol № 406 (Guinea Pig Maximisation Test

(GPMT) of Magnusson and Kligman) [2], as well as methods for "Testing of Chemicals of Human Hazard", including "Skin Sensitisation Testing" (GOST 32375-2013) [3] and "Repeated Dose Dermal Toxicity Testing. 21/28-day Study" (GOST 32642-2014) [4], etc., are widely used to assess skin toxicity.

However, the animal studies of adverse drug effects acquire more and more restrictions [5], while the *in vitro* tests based on using human cells are species-specific, show higher reproducibility and sufficient reliability [6, 7]. In particular, assessment of the eye injury and/or irritation can be performed using the reconstructed human cornea-like epithelium (EpiOcular™ (MatTek; USA), MCTT HCE™ (Biosolution; South Korea)) [8, 9], while the corrosion properties can be assessed using the reconstructed human epidermis (EpiSkin™ (L'Oréal; France), epiCS® (Phenion; Germany)) [10, 11].

Cytotoxicity testing, for example, involving the HaCaT immortalized human keratinocytes, by colorimetry or fluorimetry (MTT assay, Annexin V or trypan blue stain) represents one of the stages of the test substance biosafety testing [12]. However, cytotoxic effects may be represented not only by metabolic activity alterations or cell death, but also activation of certain signaling pathways. The use of genetically modified cells carrying the reporter genes controlled by the stress sensitive promoters is a promising approach to detection of cytotoxic effects [13–15]. For example, the KeratinoSens™ assay representing the cell line carrying a luciferase reporter gene controlled by the antioxidant response component (*AKR1C2* gene) is widely used to assess the test substances' skin sensitizing potential [16].

The transcription factors, such as NF-κB and AP-1, are involved in the cells' response to a wide range of stimuli: heavy metals, ultraviolet radiation, cytokines, infectious agents, etc., and may be of interest as biomarkers of cytotoxic effects [15, 17]. Thus, the important NF-κB transcription factor regulates transcription of proteins involved in inflammation, immune response, oxidative stress, apoptosis. AP-1 plays a key role in the cells' proliferation, differentiation, aging and death. A fluorescent biosensor based on the 3T3-L1 preadipocyte cell line, showing stable GFP expression when the NF-κB pathway was activated, was earlier used to detect the anti-inflammatory effects of the plant-derived antioxidants [18]. The previously developed cell-based model of human small intestine wall built upon the Caco-2 cell line ensured the dose-dependent detection of the NF-κB transcription factor activation under exposure to cadmium [15]. The cell-based biosensor built upon HT-29 with the regulatory element for AP-1 transcription factor and the gene encoding the mCherry fluorescent protein was successfully used for screening of heavy metal toxicity [17]. However, the cell-based sensors detecting activation of the NF-κB and AP-1 signaling pathways have not yet been studied when used to assess dermatotoxic effects during *in vitro* testing.

The study was aimed to develop the cell-based sensors built upon the HaCaT immortalized human keratinocyte cell line that express green fluorescent protein (GFP) when the NF-κB (HaCaT/NF-κB) or AP-1 (HaCaT/AP-1) signaling pathway is activated, as well as to assess their information capacity when detecting the dose-dependent cell damage.

METHODS

HaCaT cell line transduction with a lentiviral construct comprising the regulatory elements of the NF-κB/AP-1 transcription factors and the gene encoding green fluorescent protein

The HaCaT cells (CLS Cell Lines Service, 300493; Germany) were cultured in the DMEM/F12 (Gibco; USA) culture medium supplemented with 10% fetal bovine serum (Gibco; USA), 0.1% GlutaMAXTM, and penicillin/streptomycin at a concentration of 100 U/mL and 100 μg/mL, respectively (Gibco; USA), in the CO_2 incubator (MCO-20AIC Sanyo; Japan) at a temperature of 37 ± 1 °C, 90 ± 10% humidity, and CO_2 concentration of 5.0 ± 1.0%. The culture medium was replaced with fresh medium every 48 h. Upon reaching 80% confluence, the cells were dissociated with the 0.25% trypsin-EDTA solution (PanEco; Russia) and resuspended in the fresh culture medium.

The cell transduction was performed using the Cignal Lenti Reporter Assay kits (QIAGEN; USA) containing lentiviral particles with the NF-κB/AP-1-induced GFP reporter. The concentration of lentiviral particles was 2×10^7 particles/mL. For transduction, the cells were plated in the 24-well plate (Corning; USA), 4×10^4 cells per well, and incubated overnight in the CO_2 incubator (MCO-20AIC Sanyo; Japan).

After 18 h the medium was collected; 80 μL of lentiviral particles were added, which corresponded to multiplicity of vector uptake of 40, together with 6 μL of SureENTRY Transduction Reagent (QIAGEN; USA) for transfection efficacy improvement. The total volume of the solution was brought to 600 μL. The 24 h incubation was carried out in the CO_2 incubator (MCO-20AIC Sanyo; Japan). In the control well, the medium was replaced with 600 μL of complete culture medium. After that the medium with lentiviral particles was replaced with DMEM/F12 (Gibco; USA) supplemented with 10% fetal bovine serum (Gibco; USA), penicillin/streptomycin at a concentration of 100 U/mL and 100 μg/mL, respectively, and 0.1% GlutaMAXTM (Gibco; USA).

Selection of transduced cells with the NF-κB/AP-1-induced GFP reporter

Selection involved the use of puromycin antibiotic (InvivoGen; USA), to which the transduced cells were resistant. The MTT assay was used to assess puromycin resistance of the non-transduced (wild type) HaCaT cells. For that the cells were plated in the 96-well plates (Corning; USA), 2×10^3 cells in 200 μL of the medium per well, with DMEM/F12 containing 10% fetal bovine serum and GlutaMAX™ (Gibco; USA). Then 0; 0.5; 1.0; 2.0; 4.0, and 8.0 μg/mL of puromycin were added, and the cells were cultured for 10 days with the medium in the wells replaced every 96 h. The cells were examined with the Primovert phase contrast light microscope (Carl Zeiss; Germany) every day.

At the end of the exposure period, the culture medium with puromycin (experiment) or the culture medium (control) was drawn from the wells, washed with phosphate-buffered saline, pH 7.4 (PBS; PanEco, Russia), and added 200 μL of fresh complete culture medium containing 0.5 mg/mL MTT. The 2 h incubation was carried out in the MCO-20AIC CO_2 incubator (Sanyo; Japan) at a temperature of 37 °C with 5% CO_2. Then the medium was collected, washing with 200 μL of PBS was performed, and 100 μL of dimethyl sulfoxide (DMSO; Helicon, Russia) were added to each well. After the 15 min mixing on a shaker (150–200 rpm, in the dark), the optical density was measured at a wavelength of 595 nm (minus background absorbance at a wavelength of 655 nm) using the iMark microplate reader (BioRad; USA). Viability was determined using the following formula:

$$\frac{\text{OD of experimental wells — OD of the medium}}{\text{OD of control wells — OD of the medium)}} \times 100\%,$$

where OD was optical density.

Table 1. Sequences of PCR primers

Gene	Sequence of primer 1 (Forward)	Sequence of primer 2 (Reverse)
GAPDH	TCGACAGTCAGCCGCATCTTCTTT	ACCAAATCCGTTGACTCCGACCTT
NFKB1	CATGGCAGACGATGATCCC	ATTTGAAGGTATGGGCCAT
RelA	CTGTCCTTTCTCATCCCATCTT	TCCTCTTTCTGCACCTTGTC
C-JUN	ATGGTCAGGTTATACTCCTCCTC	CCTCCTGAAACATCGCACTATC

Sorting of transduced cells with the NF-κB/AP-1-induced GFP reporter

Cell sorting was accomplished by plating the transduced cells obtained by selection in the population in the Petri dish with a diameter of 100 mm (Corning; USA) or the T75 flask (Corning; USA), 4×10^5 cells per dish. Upon reaching 70–80% confluence, the medium was replaced with the medium containing the NF-κB signaling pathway activator (20 ng/mL of tumor necrosis factor alpha (TNFα), purity > 95%; Elabscience, China) or AP-1 signaling pathway activator ($Cd(NO_3)_2$ at a concentration of 10 μM). After the 24 h incubation the cells were treated with the 0.25% trypsin-EDTA solution, precipitated at 300 g for 5 min, and resuspended in 1 mL of fresh culture medium for further sorting of GFP-positive cells.

The GFP-positive cells were sorted using the BD FACSMelody™ Cell Sorter (BD Biosciences; USA). The test population of cells was determined based on the parameters of forward and side light scatter in order to avoid debris and doubles. The non-transduced cells were used as negative controls (autofluorescence control). Cell sorting resulted in obtaining the transduced cell lines with the maximum levels of fluorescent protein generated in response to induction.

Assessment of dose-dependent changes in the HaCaT transduced cell fluorescence intensity associated with the NF-κB/AP-1 pathway activation.

The transduced HaCaT cells were plated in the 96-well plate, 7×10^3 cells per well, and incubated overnight in the MCO-20AIC CO_2 incubator (Sanyo; Japan) at a temperature of 37 °C. The cells were added various concentrations of test substances: TNFα and lipopolysaccharide (LPS) (purity ≥ 99%; Servicebio, China) for NF-κB activation; $Cd(NO_3)_2$ for AP-1 activation. The effects of ultraviolet radiation (UVA) with the wavelength of 365 nm on the HaCaT/AP-1 transduced cells' fluorescence levels were also assessed. Fluorescence intensity was recorded using the Infinite M200 multimode plate reader (Tecan; Switzerland) with the excitation wavelength of 477 nm and emission wavelength of 507 nm for fluorescence and the wavelength of 600 nm for absorbance. We calculated average fluorescence intensity in the cells (minus average background fluorescence intensity with no cells) relative to the control cells with no inducers (100%). Micrographs of the intact and activated biosensor were acquired with the ZOE fluorescence microscope (Bio-Rad; USA). The resulting images were processed with the ImageJ tool (NIH; USA).

Assessing the target gene expression by polymerase chain reaction

The fluorescence intensities acquired were compared with the gene expression assessment results obtained by the real-time polymerase chain reaction (real-time PCR) method for the genes encoding various subunits of NF-κB (*RelA* — p65 subunit; *NFKB1* — p50 subunit) and AP-1 (*C-JUN*) proteins. For that RNA was extracted using the kit for column-based RNA isolation (Biolabmix; Russia) in accordance with the manufacturer's protocol, and RNA was quantified using the NanoDrop 2000c unit (Thermo Scientific; USA). The reverse transcription reaction with 1 μg of RNA was performed with the use of the MMLV RT kit (Evrogen; Russia) in accordance with the manufacturer's protocol. PCR was carried out using qPCRmix-HS SYBR+LowROX (Evrogen; Russia). Primers are provided in Table 1. *GAPDH* was used as a reference gene.

Data analysis

The results obtained were processed using the R programming language for statistical data processing. The differences between groups were determined using the Student's *t*-test with Benjamini–Hochberg adjustment. The differences were considered significant at $p < 0.05$. The data were presented as $M \pm m$.

RESULTS

HaCaT cell transduction, selection and sorting

The HaCaT cells were transduced using the Cignal Lenti Reporter Assay lentiviral construct (QIAGEN; USA) containing lentiviral particles with the NF-κB/AP-1-induced GFP reporter. Assessment of puromycin cytotoxic effects by MTT assay resulted in selection of the antibiotic exposure level (minimal concentration causing death of all original cells) corresponding to 1000 ng/mL for selection of transduced cells. Selection was carried out for 10 days, and the culture medium was replaced every three days.

During the next phase we performed selection of transduced cells after activation with 20 ng/mL TNFα for HaCaT/NF-κB and 10 μM $Cd(NO_3)_2$ for HaCaT/AP-1 for 24 h. Activation of appropriate signaling pathways resulted in the fact that the functional transduced cells started producing GFP, as detected by flow cytometry. Sorting of GFP-positive cells was accomplished by using the BD FACSMelody™ Cell Sorter (BD Biosciences; USA). The test population of cells was determined based on the parameters of forward and side light scatter in order to avoid debris and doubles (Fig. 1). The non-transduced cells were used as negative controls (autofluorescence control) (Fig. 1A, C). The transduced cell line showing maximum fluorescence levels in response to induction (gate *P1*, Fig. 1B; gate *P2*, Fig. 1D) were selected for further biosensor function assessment.

Assessing the dose-dependent changes in the HaCaT transduced cell fluorescence intensity associated with the NF-κB signaling pathway activation using TNFα and LPS

The HaCaT cells transduced with the lentiviral construct containing the NF-κB-induced GFP reporter obtained by selection and subsequent sorting were tested for the dose-dependent changes in fluorescence intensity by fluorometry using various concentrations of the well-known inducers of this signaling pathway, TNFα and LPS (hereinafter, inducers). The transduced cells were added various concentrations of inducers; then the sensor cell fluorescence intensity was

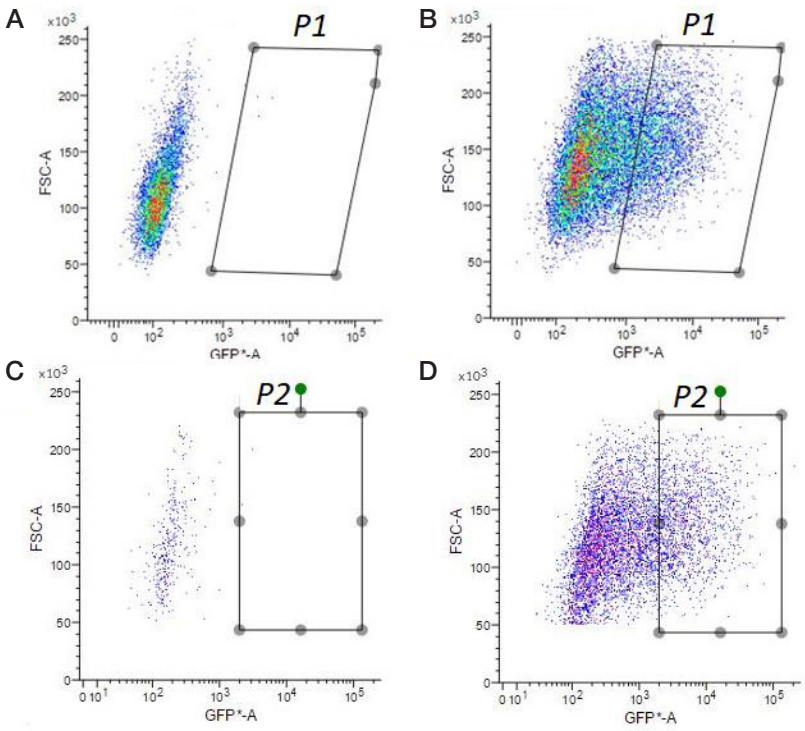

Fig. 1. Fluorescence intensity of transduced cells: control cells **(A, C)** and transduced cells showing high fluorescence intensity **(B, D)** in response to stimulation with 20 ng/mL TNFα for HaCaT/NF-κB (gate P1) and 10 μM $Cd(NO_3)_2$ for HaCaT/AP-1 (gate P2) throughout 24 h

recorded with the Infinite M200 multimode plate reader (Tecan; Switzerland), and the micrographs obtained with the ZOE fluorescence microscope (Bio-Rad; USA) were examined. Thus, to ensure NF-κB activation, the cells were added TNFα and LPS at a concentration of 0–80 ng/mL for both substances. The changes in fluorescence intensity were detected after incubation with inducers (Fig. 2). Furthermore, the NF-κB signaling pathway activation was detected based on the emergence of cells exhibiting green fluorescence when exposed to TNFα with a concentration as low as 5 ng/mL. The fluorescence intensity and the number of cells exhibiting fluorescence increased with increasing TNFα concentration (up to 10 ng/mL) (Fig. 2A, B).

Under exposure to LPS, a significant fluorescence intensity increase and, therefore, the NF-κB signaling pathway activation were also detected at the minimum test concentration of 5 ng/mL. The relationship between the fluorescence intensity and the concentrations of both inducers used reached the plateau when the concentration exceeded 20 ng/mL (Fig. 2C, D).

The fluorescence intensity measurement results were compared with the results of assessing the expression of genes encoding various NF-κB protein subunits (*RelA* — subunit p65;

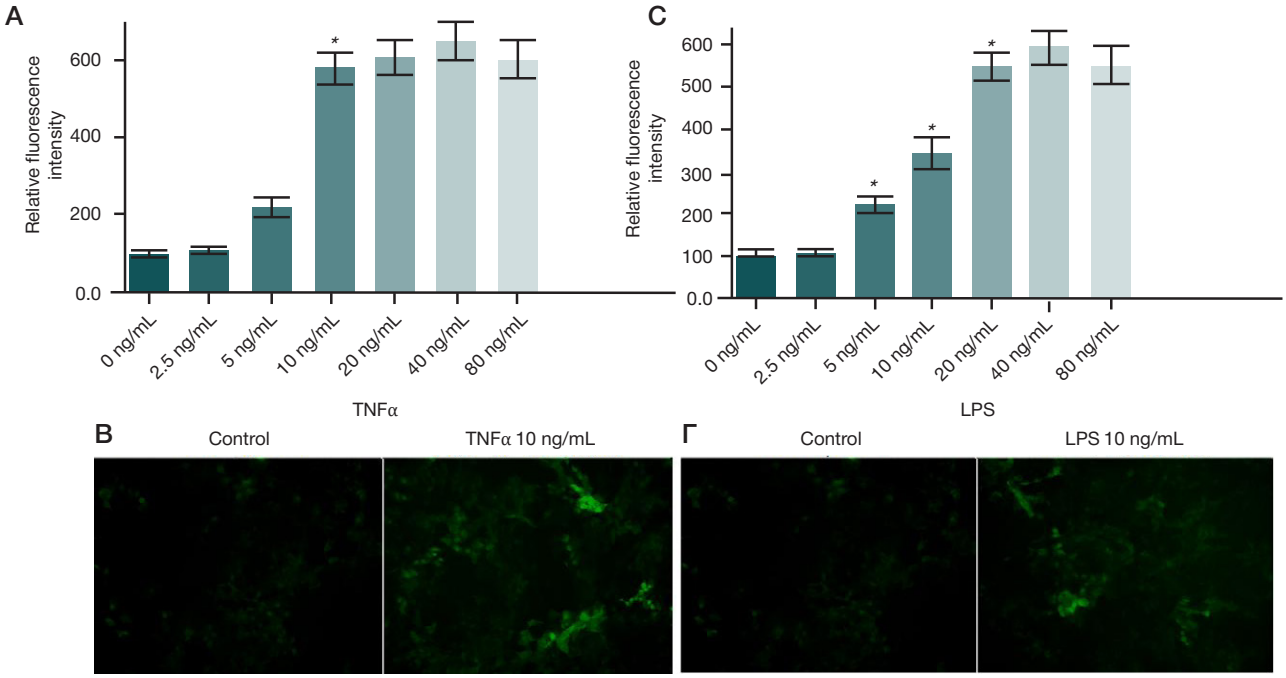

Fig. 2. Changes in the HaCaT/NF-κB cells' relative fluorescence intensity under exposure to TNFα **(A, B)** and LPS **(C, D)**. Fluorometry results **(A, C)** and fluorescence microscopy **(B, D)**. * — significant differences from the previously reported concentration, $p < 0.05$.

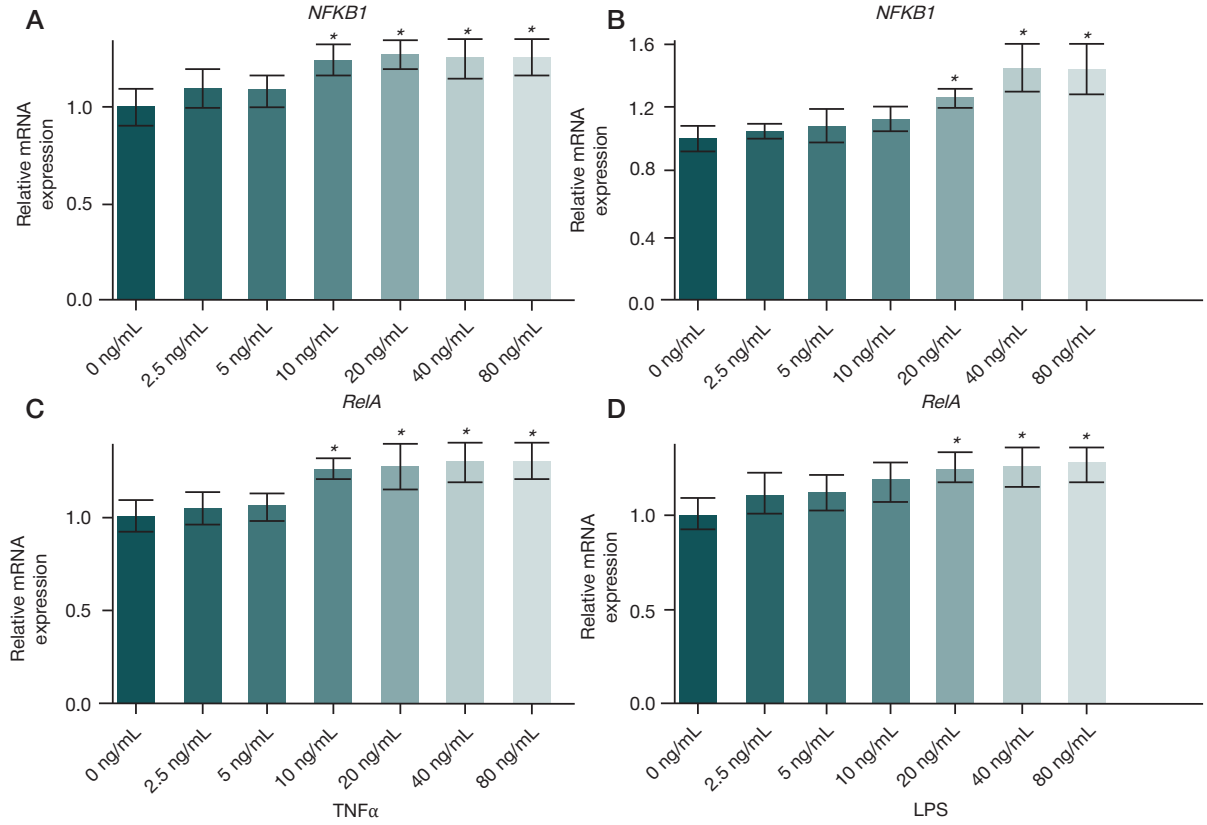

Fig. 3. Assessing activation of the genes encoding the NF-κB transcription factor subunits. Relative expression of *RelA* and *NFKB1* mRNA under exposure to TNFα **(A, B)** and LPS **(C, D)**. * — significant differences from the control group, $p < 0.05$

NFKB1 — subunit p50) (Fig. 3). It was shown that exposure to TNFα resulted in the increased expression of *RelA* and *NFKB1* mRNA, however, significant differences from controls were obtained for the concentration of 10 ng/mL only, without further expression increase (Fig. 3A). A similar trend was observed under exposure to LPS (Fig. 3B): a significant increase in the *RelA* and *NFKB1* mRNA expression was observed only under exposure to LPS at a concentration of 20 ng/mL, without significant changes in expression with further concentration increase.

Assessing the dose-dependent changes in the HaCaT transduced cell fluorescence intensity associated with the AP-1 signaling pathway activation

We used $Cd(NO_3)_2$ and UVA, the inducers causing redox imbalance in the cells, to estimate information capacity of the cell-based sensor enabling detection of the AP-1 signaling pathway activity. We recorded the biosensor fluorescence intensity as a function of the $Cd(NO_3)_2$ concentration or radiation intensity (Fig. 4; Table 2). The findings about the AP-1 pathway activation were compared with the results of assessing the expression of gene *C-JUN* encoding the AP-1 protein subunit.

It was shown that exposure to $Cd(NO_3)_2$ resulted in the increase in fluorescence exhibited by the HaCaT/AP-1 cells, when the concentration was between 20 μM and 40 μM. However, the changes in fluorescence were not dose-dependent. Perhaps, the cytotoxic effects of this inducer manifested themselves, when the $Cd(NO_3)_2$ concentration increased, which resulted in the decrease in the number of viable cells exhibiting fluorescence. Furthermore, the *C-JUN* mRNA expression increased under exposure to the concentration as low as 5 μM, however, it did not depend on the exposure dose when the concentration increased (Table 2; Fig. 4A).

We also assessed the sensor cells' response to the 24 h exposure to various doses of ultraviolet radiation with the wavelength of 365 nm. The fluorescence intensity measurement results were also compared with the results of assessing the expression of gene *C-JUN* encoding the AP-1 subunit. A significant increase in the sensor cell fluorescence intensity was observed when the UV radiation exposure dose was 12–18 J/cm^2 (significant compared to control cells). The fluorescence intensity significantly differed from control when the exposure dose increased up to 18 J (Table 2; Fig. 4B). At the same time, the increase in the *C-JUN* mRNA expression was observed, when the cells were exposed to the studied UVA doses. However, the changes were not dose-dependent.

Table 2. Changes in the HaCaT/AP-1 cell fluorescence associated with exposure to $Cd(NO_3)_2$

$Cd(NO_3)_2$, μM	Relative fluorescence intensity, %	UVA, J/cm^2	Relative fluorescence intensity, %
0	100 ± 13	0	100 ± 16
5	115 ± 18	6	135 ± 18
10	121 ± 16	12	168 ± 21*
20	172 ± 49*	18	151 ± 24*
40	166 ± 43*		

Note: * — significant differences from the control group, $p < 0.05$

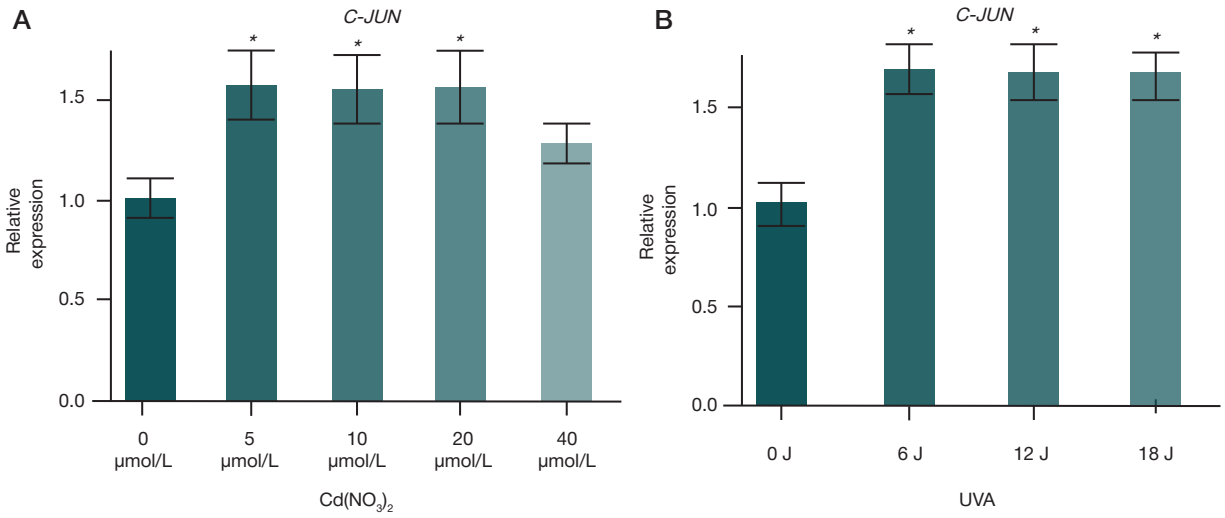

Fig. 4. Assessing activation of the gene encoding the AP-1 transcription factor subunit. Relative expression of the C-JUN mRNA under exposure to $Cd(NO_3)_2$ **(A)** and UVA **(B)**. * — significant differences from the control group, $p < 0.05$

DISCUSSION

The development of *in vitro* tests for estimation of the chemical compound (CC) biosafety is an urgent task, since nowadays the animal studies become increasingly constrained [5, 19]. Keratinocytes are the first to contact the damaging agents and are involved in immune response, therefore, they represent a promising screening model for dermatological effects. Furthermore, the HaCaT immortalized keratinocyte cell line is a convenient alternative to primary cells for *in vitro* toxicology studies [12]. Thus, the HaCaT cells demonstrate normal morphogenesis and express all surface markers of primary keratinocytes. These cells can also differentiate when stimulated and express specific differentiation markers, such as keratin 14, keratin 10, and involucrin. Furthermore, the HaCaT cells can switch between the differentiated and basal states due to changes in the Ca^{2+} concentration in the culture medium [20, 21]. However, it is important to consider the presence of GOF (gain-of-function) mutations in the gene *TP53* resulting in the increased proliferation rate and abnormal terminal differentiation, when using HaCaT cells [22]. Nevertheless, in contrast to primary keratinocytes, the HaCaT cells do not require growth and/or differentiation factors in the culture medium, have boundless proliferation potential, and demonstrate a stable phenotype regardless of the number of passages [23]. Thus, the HaCaT cell line is a promising experimental model for investigation of various physiological processes occurring in human keratinocytes, including when assessing the CC toxicological effects.

When developing test systems for identification of irritation, providing the possibility of testing specific biomarkers associated with injury is considered to be promising [6, 24]. This makes it possible to draw a conclusion about the test substance potential cytotoxicity and the molecular mechanisms underlying realization of cytotoxicity.

Today, the focus is on the approaches aimed at studying the early cellular response to damage, preferably by real-time monitoring. Such approaches represent a promising alternative to the routine measurement of finite exposure effect, for example, based on the detection of specific metabolic processes using colorimetric and fluorometric assays (MTT assay, trypan blue stain, etc.). Thus, today the tests enabling the high-throughput real-time analysis of the cell damage process dynamics, such as STACK (scalable time-lapse analysis of cell death kinetics), are well known [25]. In particular, fluorescence is used for identification of the population of viable and dead cells by fluorescence microscopy and thorough optimization of the image analysis procedures. However, the cytotoxic effects not only include cell death, but can be associated with the skin sensitization, one of the key events in the development of which, according to OECD-approved tests, is represented by activation of specific signaling pathways in keratinocytes (such as Keap1/Nrf2-ARE, NF-κB, etc.) [26].

To assess potential damaging effects of the test substances in the cellular response tests when assessing their potential biosafety at the molecular biological level, in this study we developed the cell-based sensors built upon the HaCaT immortalized human keratinocytes having regulatory elements for the NF-κB and AP-1 transcription factors and the gene encoding green fluorescent protein (GFP). After the delivery of these genetic constructs, the cells produced GFP, when appropriate signaling pathway was activated. In this case, fluorescence intensity was quantified. Despite the fact that peak GFP expression in the cells is achieved 24 h after the event detected using the protein, the GFP expression dynamics is well understood and accurately described. In this regard, the cell-based sensors built upon GFP make it possible to assess the dynamics of cellular response to the test exposure. In this case, it is necessary to consider the delayed effect of fluorescence and the cellular event itself [15].

Despite the fact, that the NF-κB and AP-1 transcription factors are among the most abundant actors of the cell damage process realization at the molecular level, the instrumental assessment performed has shown various possibilities of using the HaCaT/NF-κB and HaCaT/AP-1 sensors developed for detection of the dose-dependent effects of the well-known inducers of appropriate signaling pathways in the cells. The acquired cell-based sensor with the reporter construct associated with NF-κB activation demonstrated the possibility of sensitive dose-dependent *in vitro* detection of this signaling pathway activity in the model human epidermal cells. The GFP expression level of the biosensor construct associated with activation of the NF-κB signaling pathway strongly correlated with the changes in expression of target mRNA measured by real-time PCR. In turn, the HaCaT/AP-1 cell-based sensor made it possible to fix the fact of appropriate signaling pathway activation by inducers, however, detection of the inducer dose-dependent effects turned out to be impossible.

It is also worth noting that the information capacity of the testing results obtained using both sensors decreased with

increasing concentration of the inducers employed. This was probably due to a significant decrease in the number of viable sensor cells under these conditions, which did not allow to correctly estimate the fluorescence measurement results. In this regard, it is reasonable to perform MTT assay or other similar assay allowing one to determine the test substance concentrations causing death of sensor cells before conducting the tests involving the HaCaT/NF-κB and HaCaT/AP-1 sensors. This will make it possible to more correctly estimate early cellular events occurring in response to the effects of test substances, including subtoxic ones.

CONCLUSIONS

In this study we developed biosensors based on the HaCaT immortalized keratinocytes containing genetic systems involved in activation of the GFP fluorescent reporter protein, the dose-dependent increase in expression of which was associated with activation of the AP-1 and NF-κB signaling pathways associated with damage to human epidermal cells. Experimental detection of the developed biosensors' sensitivity was conducted. We assessed the possibility of quantifying the intensity of sensor cell activation in response to exposure to inducers. It was found that the changes in HaCaT/NF-κB fluorescence was observed under exposure to low concentrations of TNFα or LPS, which was in line with the changes in expression of genes *RelA* and *NFKB1* and had a dose-dependent nature. The cells with the HaCaT/AP-1 biosensor also responded to the $Cd(NO_3)_2$ and UVA exposure by increasing the fluorescence intensity and the target gene expression, however, we failed to detect the dose-dependent effects of these inducers on the sensor cells. The cell-based sensors developed can be used for *in vitro* assessment of cytotoxic effects of the test substances on human skin cells, as well as for fundamental studies of cytotoxicity mechanisms. Moreover, the HaCaT/NF-κB sensor seems to be the most promising in terms of the possibility of detecting the dose-dependent effects of the damaging substances.

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