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SEARCH FOR BIOLOGICAL MECHANISMS OF TOXIC ACTION OF SHOE GLUES: CELL VIABILITY IN VITRO, ALBUMIN DAMAGE AND FREE RADICAL GENERATION

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Key words: *shoe glues, in vitro method, cytotoxicity, conformational changes of albumin, radicals activity*
Ключові слова: *взуттєві клеї, метод in vitro, цитотоксичність, конфірмаційні зміни альбуміну, радикальна активність*

Abstract. Search for biological mechanisms of toxic action of shoe glues: cell viability *in vitro*, albumin damage and free radical generation. Lototska-Dudyk U.B., Kuzminov B.P., Lototska L.B., Klyuchivska O.Yu., Stoika R.S. *The combined use of cellular, extracellular research methods for studying the toxic effect of shoe glues is an additional tool for screening and assessing the potential risks of their use. The aim of the work was to investigate the mechanisms of the toxic effect of shoe glues at the cellular, molecular and biochemical levels. Rubber, polychloroprene and polyurethane shoe glues were used. Three experimental approaches were applied: measurement of survival of mammalian cells, a spectroscopic study of conformational changes of albumin, and a free radical measurement. Cytotoxicity testing was performed on murine fibroblasts Balb/c-3T3 line, human embryonic kidney cells HEK-293 and human keratinocytes of the HaCaT line treated for 24 and 72 hours with glues samples. A survival of treated cells was monitored using MTT-test. Changes in the spectral characteristics of albumin were monitored during exposure for 24 hours and 21 days with "fresh" and "dried" samples of glues. Content of free radicals was evaluated in the reaction with DPPH reagent. The cytotoxicity was increased with increasing exposure time, and depended on both the type of glue and the type of treated cells. The polyurethane glue demonstrated the most pronounced cytotoxic effect. Balb/c-3T3 fibroblasts were the most sensitive to the action of all types of glues, a reliable maximum increase in cell death was manifested in 72 hours exposure (28.9-19.1% of living cells).While cells of HEK-293 and HaCaT lines were more resistant. At 24 hours contact, their viability was 99.12-79.22% and 99.0-56.9%, respectively. Increased exposure up to 72 hours reliably caused a decrease in the survival of these cell lines – 96.24-68.1% and 82.2-51.7%. The loss of the solvent didn't affect the cytotoxic effect of the studied glues. Conformation changes in albumin were manifested during its long-term contact with both "fresh" and "dried" glues. Manifestations of the toxic effect of glues on biomolecules were increased in the sequence: rubber > polyurethane > polychloroprene. Shoe glues demonstrated an ability to generate free radicals in the sequence: rubber > polychloroprene > polyurethane. These manifestations were increased in a time period of 4 hours – 24 hours. That may create risks when they are used. The results can be used to determine the targets and mechanisms of the toxic effect of shoe glues and to obtain new knowledge in the field of research of industrial toxicants.*

Реферат. Пошук біологічних механізмів токсичної дії взуттєвих клеїв: життєздатність клітин *in vitro*, ушкодження альбуміну та генерація вільних радикалів. Лотоцька-Дудик У.Б., Кузьмінов Б.П., Лотоцька Л.Б., Ключівська О.Ю., Стойка Р.С. *Метою роботи було дослідити механізми токсичної дії взуттєвих клеїв на клітинному, молекулярному та біохімічному рівнях. Досліджували каучуковий, поліхлоропреновий та поліуретановий взуттєві клеї. Було застосовано три експериментальні підходи, а саме: тест на виживання клітин ссавців in vitro, спектрофотометрична оцінка модифікації альбуміну та дослідження радикальної активності. Цитотоксичність вивчено на фібробластах миші лінії Balb/c-3T3, ембріональних клітинах нирки людини лінії HEK-293 та людських кератиноцитах лінії HaCaT, оброблених впродовж 24 і 72 годин зразками клеїв. Виживаність оброблених клітин контролювали за допомогою MTT-тесту. Зміни спектральних характеристик альбуміну відстежували при експозиції 24 год і 21 доба 1% розчину цього білка на свіжий і сухий клеї. Оцінювання радикальної активності проведено за допомогою DPPH-реактиву. Цитотоксичність клеїв зростала зі збільшенням експозиції та залежала як від виду клею, так і типу клітин. Найбільш виражену цитотоксичну дію проявив поліуретановий клей. Клітини сполучної тканини лінії Balb/c-3T3 були найбільш чутливими до дії усіх видів клеїв, достовірне максимальне збільшення загибелі клітин проявилося за 72 год експозиції (28,9-19,1% живих клітин). Клітини епітеліальної тканини лінії HEK-293 та HaCaT виявилися більш стійкими. За 24 год контакту їхня життєздатність становила 99,12-79,22% та 99,0-56,9% відповідно. Збільшення експозиції до 72 год достовірно спричинило зниження виживання цих клітинних ліній – 96,24-68,1% та 82,2-51,7%. Втрата розчинника не вплинула на цитотоксичний ефект досліджуваних клеїв. Токсична дія клеїв на біомолекули альбуміну зростала в послідовності: каучуковий > поліуретановий > поліхлоропреновий при тривалому контакті як зі свіжими, так і висушеними клеями. Взуттєві клеї генерували вільні радикали в послідовності: каучуковий > поліхлоропреновий > поліуретановий. Прооксидантні прояви достовірно зростали в часовому періоді 4 год–24 год, що при екстраполяції результатів у реальні умови створює ризики їх застосування. Результати можуть бути використані для визначення мішеней та механізмів токсичної дії взуттєвих клеїв та отримання нових знань у галузі дослідження промислових токсикантів.*

The extensive list of chemicals used in footwear manufacturing technology necessitated an in-depth study of the toxicity of both separate substances and their compounds. Glue compounds, the use of which involves direct contact of workers with them, deserve special attention in this aspect [1].

The most common glues in footwear manufacturing technology are adhesives-solutions, adhesives-dispersions and hot-melt adhesives [2, 3]. The glue consists of an adhesive-active polymer that provides bonding strength and additional components of various functional purposes (solvents, vulcanizing agents (hardeners), fillers, stabilizers, pigments, and others) [4]. Numerous publications [5, 6, 7, 8] confirm the negative effect and manifestations of toxic effects, even at low levels, of shoe glues of these groups on the workers in production conditions. This activates the issue of in-depth research into the mechanisms of toxicity of shoe glues and their

components and is an additional screening tool for industrial pollutants.

A traditional and generally accepted approach to assessing the degree of toxicity of chemical substances is the use of warm-blooded animals in toxicological experiments. However, the high resource requirements for standard toxicological studies, the introduction of new scientific technologies create wide opportunities for the use of more modern methods at the cellular and extracellular levels in toxicological testing [9].

Alternative test models complement, or in some cases can replace, classical paradigms for hazard identification and risk assessment [10, 11]. It also supports reducing reliance on animal testing and following the 3R approach (replace, reduce and refine) [12, 13].

It is clear that one biotest cannot detect all mechanisms of toxicity; the use of a set of biotests will allow to obtain more information about the degree of toxicity and danger of chemical substances and their compounds.

Therefore, the aim of the work was to investigate the mechanisms of the toxic effect of shoe glues at the cellular, molecular and biochemical levels using alternative methods.

MATERIALS AND METHODS OF RESEARCH

For research, the following shoe glues were used: rubber glue-dispersion Bonidur LZT-4 (sample 1), polychloroprene glue-solution Boterm GTA-1 (sample 2) and polyurethane glue-solution Bonikol Pur (sample 3), which are most actively used in shoe manufacturing technology. Dispersion adhesives (latexes) are highly concentrated aqueous dispersions of chloroprene rubber. The toxicity of these glues is due to the presence of chloroprene monomer residues and its decomposition products. The main components of glues-solutions are an adhesive (polyurethane or polychloroprene rubber) in a mixture of organic solvents (acetone, toluene, ethyl acetate, trichlorethylene, gasoline) and a hardener (isocyanates).

The following *in vitro* methods were used in the study:

Cultured cells. The cytotoxic effect was studied on three mammalian cell lines (pseudonormal human embryonic kidney cells of HEK-293 line, pseudonormal human keratinocytes of the HaCaT line and murine fibroblasts of Balb/c-3T3 line). The cells were taken from the Cell Bank of the department of regulation of cell proliferation and apoptosis of Institute of Cell Biology of the National Academy of Sciences of Ukraine (Lviv). Cell culturing was carried out in the Dulbecco-modified Eagle's medium (DMEM) (Sigma Chem Co., USA) in the presence of 10% fetal bovine serum (Sigma Chem Co., USA) and 40 μg/ml gentamicin (Sigma, Chem Co., USA) in a thermostat with 5% CO_2 content at 37°C and 100% humidity. Cells were sub-cultured every 3 days in a ratio of 1:5.

Cells were seeded in plastic 24-well plastic dishes at 10^5 concentration of cells per well. 24 hours later tested samples of shoe glues (fresh and dried) were introduced. Native cell culture was used as a control. Doxorubicin (Dox.) (Kyivmedpreparat, Ukraine) at a dose of 5 μg/ml as a toxicity control and DMSO (Dimethyl sulfoxide) at a dose of 10 μg/ml as an irritant factor control were additionally compared drugs. The experimental exposure time was 24 and 72 hours.

Examination of experimental cells and assessment of living, apoptotic and dead cells was carried out on a DeltaOptical (China) light fluorescent inverted microscope at a magnification of ~400 times in the excitation range of 320-570 nm and emission range of 420-750 nm.

MTT-assay. The number of living cells was determined with the MTT (methylthiazolyldiphenyltetrazolium bromide), using the manufacturer's recommendations (Sigma, Chem Co., USA) [14]. The measurement principles are based on the ability of the mitochondrial dehydrogenases of living cells to reduce colorless forms of the MTT-reagent [3- (4.5-di-methylthiazol-2-yl) – 2.5-dimethyl bromide tetrazolium, Sigma-Aldrich, USA)] to blue crystalline formazan which is soluble in the DMSO.

After 1-3 hours of incubation of cells with MTT, DMSO was added to the wells to dissolve the formazan crystals and quantified on a multichannel microphotometer Plate Reader BioTeк 76883 (ELx800 Absorbance Reader (BioTek, USA) at a wavelength of 540 nm. The number of living cells directly depends on the optical density (extinction) of the formed formazan, which was expressed as a percentage relative to the control which was taken as 100%.

Spectroscopic study of conformational changes of albumin. At a search of the potential molecular target(s) of toxic action of studied glues, we used albumin that is known to be one of the most abundant proteins in human body. The action of toxicants caused conformational changes in this protein which affected its ability to absorb UV rays with a maximum at ~ 278 nm and changed spectrum profiles in this range in the case of chemical transformations. As an experimental model, 1% solution of human serum albumin (HSA) was used (Reanal, Hungary). Changes in the spectral characteristics of albumin were monitored using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific) (USA) [15] at exposure for 24 hours and 21 days with "fresh" and "dried" samples of adhesives.

Free radical measurement. Content of free radicals was evaluated in the reaction with DPPH reagent

(2.2-Diphenyl-1-picrylhydrazyl) (Alfa Aesar, Germany). DPPH is used to assess the activity of free radical scavenging by natural or synthetic compounds [16]. To measure content of free radicals, samples of studied glues in the DMSO were introduced into the wells of a 96-well plastic dish in the amount of 10 µl per well.

The DPPH reagent was prepared as a 0.01% solution in the DMSO. The reagent was introduced into wells with tested glues immediately before the beginning of the measurement. For measurement of optical density, a multichannel microphotometer Plate Reader BioTek 76883 (BioTek, USA) at 490 nm was used. It was measured after 30 min, 4 hours, 1, 3, 5, 7 days of the experiment. The percentage of change in the solution's optical density was determined based on the formula:

$$(\%) = [(A_0-A_1)]/(A_0)]\times 100,$$

where:
A_0 – adsorption of the control sample; A_1 – adsorption of the tested sample.

The DMSO solvent was used as a zero control, while 5 µg of ascorbic acid was an antioxidant control, and 1 µg of doxorubicin was a pro-oxidant control.

The study was conducted without experiments on humans or animals, which is confirmed by the minutes of the meeting of the Board of Bioethics at Danylo Halytsky Lviv National Medical University, protocol № 1, dated January 15, 2024.

Statistical analysis. The obtained digital data were processed using the method of variational statistics using the STATISTICA 6.1 (StatSoft Inc., serial № AGAR909E415822FA).

The hypothesis of normal distribution of the studied indicators was tested using the Shapiro-Wilk test. The probability of the difference between the control and experimental measurements was assessed using the Student's t-test. All data are presented as the M±m (arithmetic mean ± standard deviation), relative (%) values. P values less than 0.05 were considered statistically significant and marked with asterisk: *p<0.05 [17].

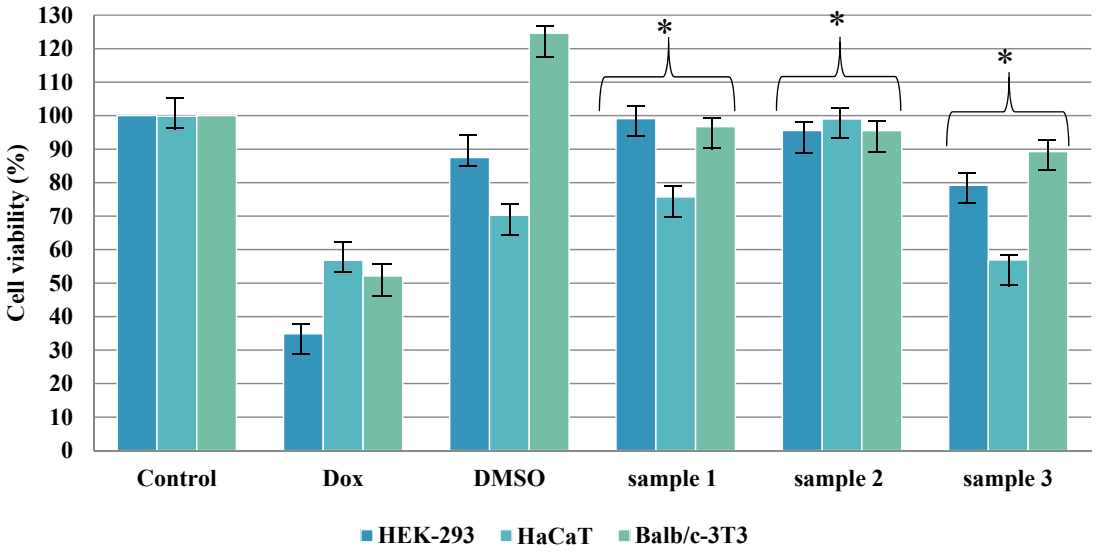
RESULTS AND DISCUSSION

The cytotoxicity of glues was increased with prolongation of the exposure time and depended on both the type of cells and the type of glue (Fig. 1 a, b).

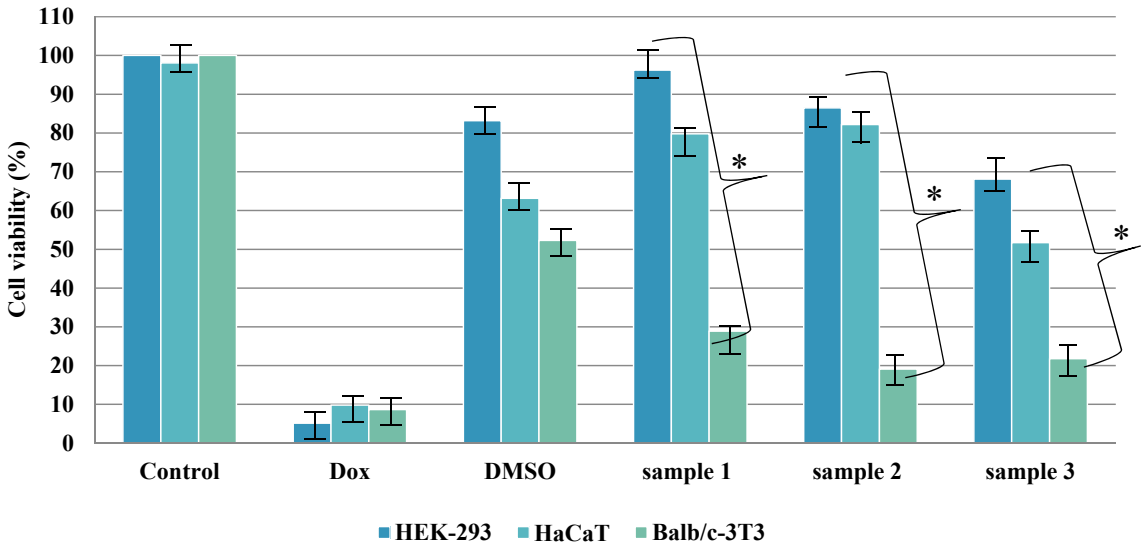
After 24 hours of contact, cells of the Balb/c-3T3 and HEK-293 lines were reliably sufficiently resistant to the action of all samples of "fresh" glues. Their viability was 96.7-89.26% and 99.12-79.22%, respectively. The viability of HaCaT cell line during these experimental periods was 99.0% (sample 2) and 75.7%; 56.9% (samples 1 and 3).

After 72 hours of contact, a significantly (p<0.05) pronounced cytotoxic effect of all samples of "fresh" glues was observed against Balb/c-3T3 fibroblasts. Only 28.9%, 19.1%, and 21.76% of cells remained viable, respectively. Other cell lines were more resistant to the toxic effects of "fresh" glues.

The viability of HEK-293 and HaCaT cell lines was moderately reduced to 96.24-68.1% and 82.18-51.7%, respectively. The most pronounced cytotoxic effect for these cell lines was demonstrated by the sample of "fresh" glue 3.



a) "Fresh" shoe glues 24 hours.

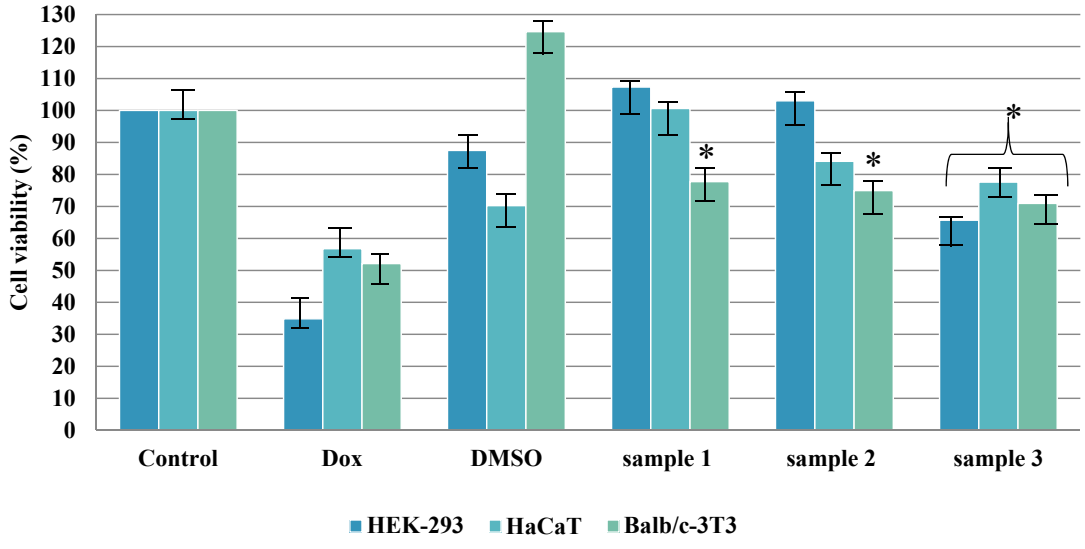


b) "Fresh" shoe glues 72 hours.

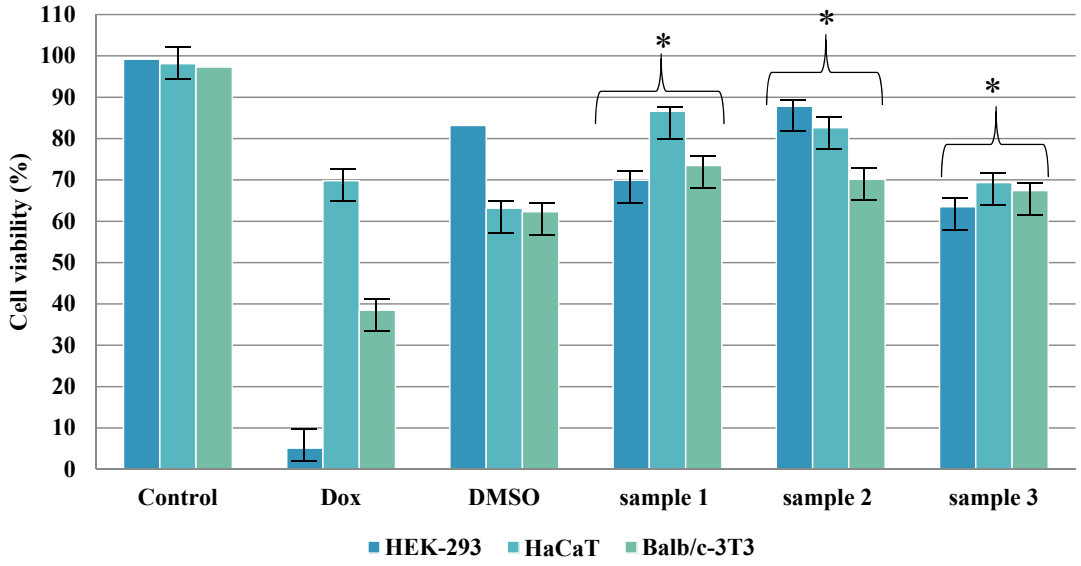
Note: * p<0.05 - significance of difference compared to non-treated control cells.

Fig. 1. Cell viability (%) after incubation with "fresh" shoe glues

However, despite the absence of a solvent, "dried" glues also had a damaging effect on cultured cells (Fig. 2 a, b). The viability of Balb/c-3T3 cells ranged from 70.96 to 77.7% after 24 hours of contact regardless of the type of glue. A significant decrease in the viability of HEK-293 (65.7%) and HaCaT (77.6%) cell lines was recorded under the influence of only sample 3 of "dried" glue. The recorded proliferative effect on HEK-293 cells (107.34%; 103.0%) and the viability of HaCaT cells (100%; 84.12%) of glue samples 1 and 2 turned out to be not significant ($p>0.05$). At 72 hours exposure, the cytotoxicity of glues was characterized by a moderate increase. The viability of Balb/c-3T3 cells was decreased to 73.51-67.4% (samples 1-3), while the viability of HaCaT cells was decreased to 86.6-69.4% (samples 1-3), and the viability of HEK-293 cells was decreased to 69.9-63.5% (samples 1, 3), 87.8% (sample 2).



a) "Dried" shoe glues 24 hours



b) "Dried" shoe glues 72 hours

Note: * p<0.05 – significance of difference compared to non-treated control cells

Fig. 2. Viability of cells (%) after incubation with "dried" shoe glues

The effect of shoe glues towards a protein of human plasma (Fig. 3) showed the absence of changes in the spectral characteristics of albumin in the far UV region and at 278 nm at 24 hours exposure after the action of both "fresh" and "dried" glues.

However, on the 21st day of the experiment, several new absorption peaks were detected under the action of "fresh" glue that might be caused by the formation of new bonds between separate functional groups of amino acids of the polyunsaturated chain. At the action of "dried" glue on the 21st day, the denaturing effect towards albumin was more pronounced, which was manifested as a significant decrease in the absorption of albumin. This indicates a direct deleterious effect of sample 1>sample 3>sample 2 towards albumin.

We found that the components of shoe glues can disrupt the antioxidant/pro-oxidant balance and cause an oxidative stress (Fig. 4).

We found that "fresh" glues a significantly ($p<0.05$) possessed pro-oxidant action in 4 hours experiment. Its intensity was increased on the 1st day (24 hours) of the experiment: sample 1 (-2.4 CU → -16 CU), sample 2 (-1.2 CU → -7.5 CU), however, later on, this activity changed to the opposite. The pro-oxidant properties of the sample of "fresh" glue 3 were recorded only in 4 hours experiment (-2.0 CU), and later, didn't show signs of stimulation of the development of free radicals during the whole period of the experiment. A significant absence of pro-oxidant properties was recorded for the "dried" glues: sample 1 (exposure 30 min – 120 h), sample 2 (exposure 30 min – 24 h) and sample 3 (whole period of the experiment). In other time periods, these manifestations were not significant ($p>0.05$).

The research results prove the necessity of an in-depth study of the toxicity mechanisms of industrial chemical compounds. This work confirms the use of test objects of various biological organizations under *in vitro* conditions to assess chemical toxicity and health risks [18, 19]. Human and animal cells cultures are the most appropriate since they represent a highly balanced homeostatic mechanism that responds to external stimuli in different ways [20]. Experiments conducted directly on human cell cultures simplify the extrapolation of toxicity prediction data to the human organism, which explains the selection of these cell lines.

The choice of epithelial and connective tissue cell cultures is determined by the pathway of exposure to the components of shoe glues in workers under production conditions. The detected cytotoxic effect of "fresh" glues is primarily due to the presence of organic solvents. The most pronounced cytotoxic effect was observed in Balb/c-3T3 cell lines, which is consistent with the findings of other researchers [21] regarding the cytotoxicity of organic solvent mixtures (benzene, toluene, ethyl acetate, etc.) towards fibroblasts and other cell targets. It was found that the effect of low (non-toxic) concentrations is additive, and a total toxic effect must be taken into account when establishing the occupational risk parameters. Other studies [22] indicated that the cytotoxicity of organic solvents towards cells of Balb/c-3T3 line was increased with elevation of solvent concentration.

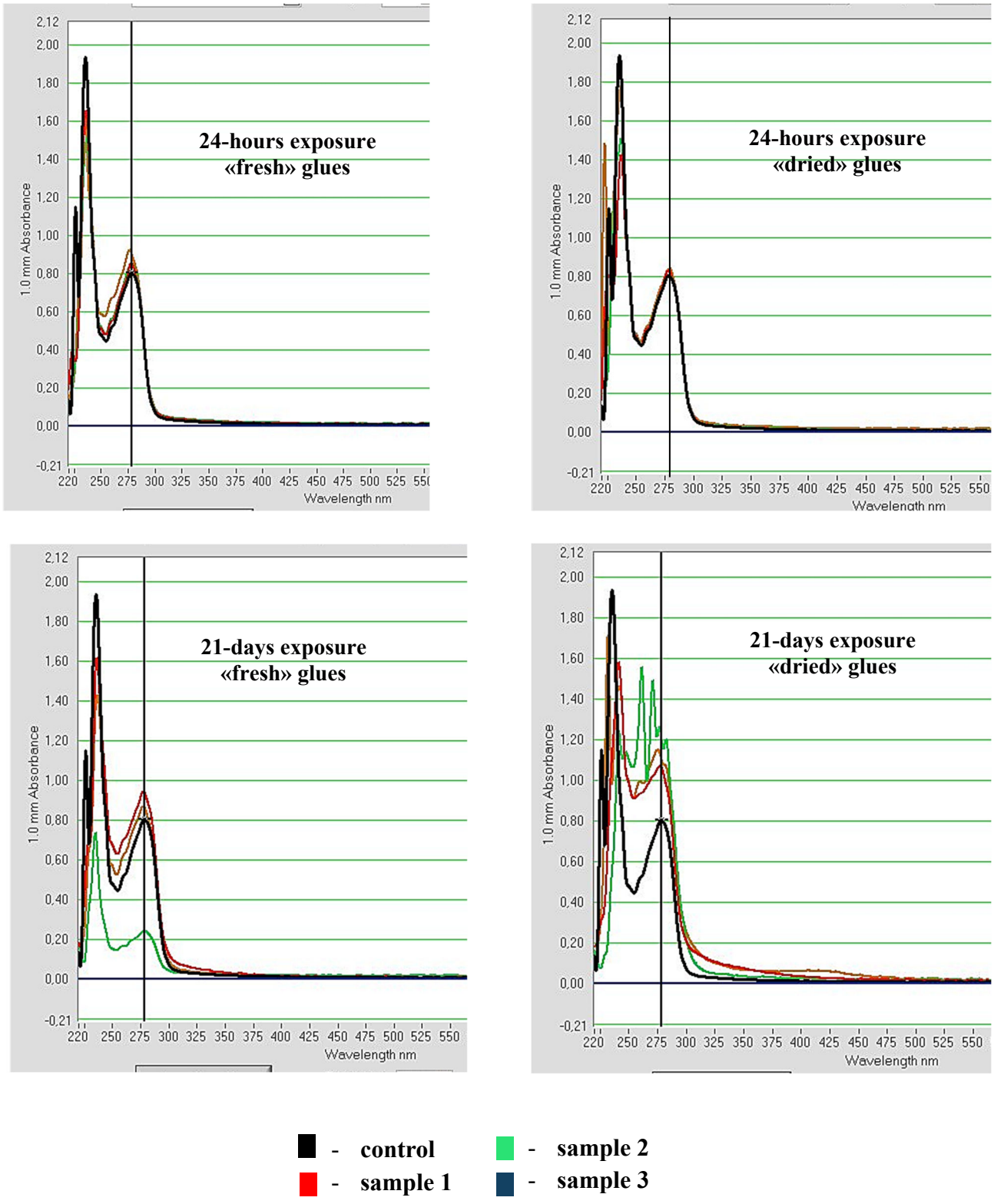
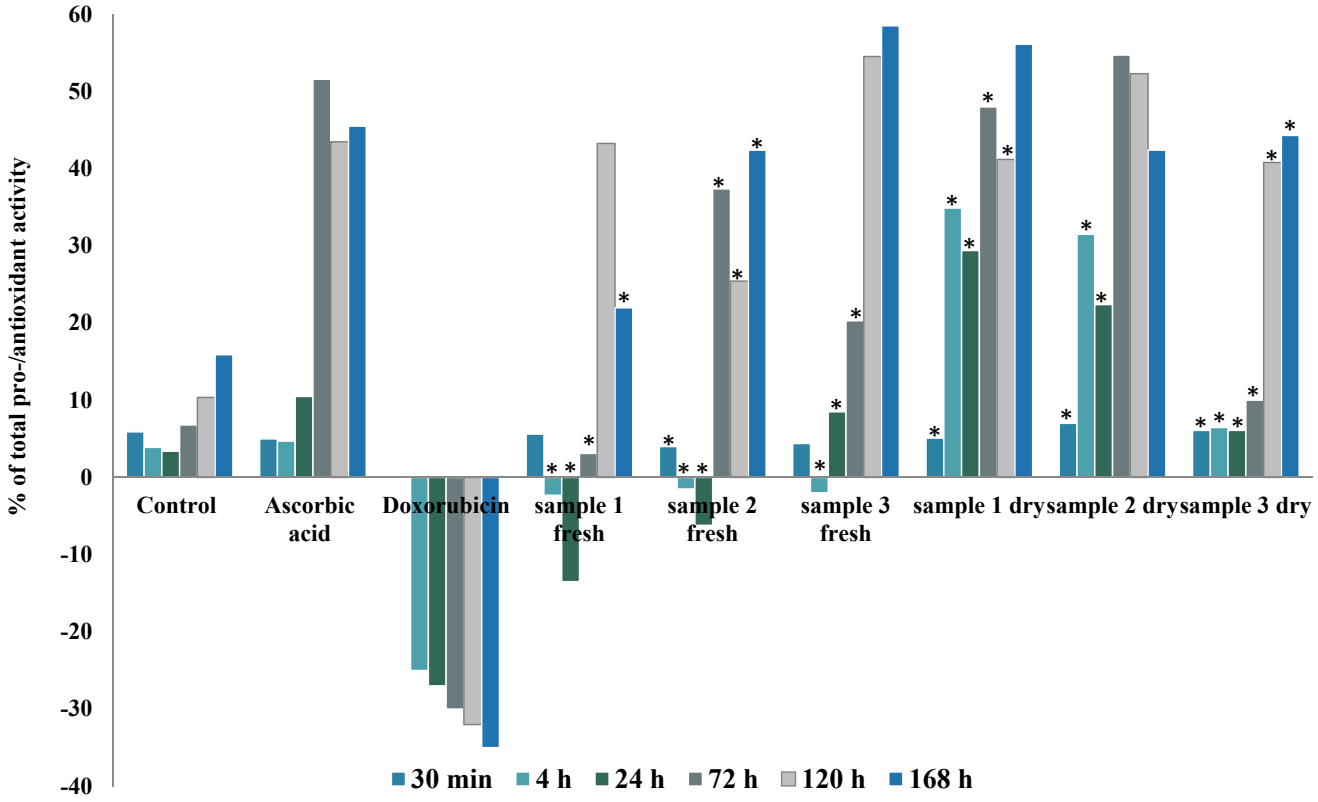


Fig. 3. Changes in spectral characteristics of 0.1% HSA under the action of "fresh" and "dried" shoe glues at different time exposure



Note: * p<0.05 – significance of difference compared to non-treated control cells.

Fig. 4. Evaluation of the pro-/antioxidant activity of shoe glues

The cytotoxic effect of "dried" glues might be explained by the presence of other biologically active components that cause this effect. The cytotoxicity of isocyanates used in glues as hardeners, especially for polyurethane raw materials, has been confirmed [23, 24]. Researchers [25] indicate the following differences in the toxic reaction of different types of cells for the isocyanate monomer: keratinocytes were the most resistant, melanocytes were the most sensitive, while fibroblasts showed an intermediate sensitivity.

Special attention should be given to studying the toxicity mechanism at the molecular level [11, 26]. Changes in the optical density of the HSA solution upon exposure to shoe glues indicate structural disruption (denaturation) of the protein. Our results agree with the data of other researchers [27, 28] who detected that the influence of foreign substances on HSA caused structural and functional changes in this protein due to denaturation and a loss of its biological activity. The interaction of serum proteins with pollutant molecules directly affects a degree and duration of the action of toxicants in the body [29] which makes it possible to elucidate the process of their metabolism *in vivo*.

The impact of shoe glues components on oxidative stress development is confirmed by the research findings of other authors [30]. The DPPH method used in this study makes it possible recording of the rate of free radical-dependent reactions, in particular with organic radicals [31] that confirms its use for evaluating the oxidation potential of shoe glues. The studied rubber glue dispersion and polychloroprene glue solution exhibiting pro-oxidant properties can cause the generation of free radical molecules. These manifestations were increased at 4 hours to 24 hours terms that may create the risks related to their use.

Thus, a combined use of alternative research methods (cellular, extracellular) can become a potentially useful tool for obtaining new knowledge in the field of research on industrial toxicants.

CONCLUSIONS

1. The in vitro cellular models for studying the toxic effect of industrial toxicants (shoe glues) might be a useful tool for screening and assessing the potential risks of their application.
2. Balb/c-3T3 fibroblasts were the most sensitive to the action of all types of glues, a reliable maximum increase in cell death was manifested in 72 hours exposure (28.9-19.1% of living cells).While cells of HEK-293 and HaCaT lines were more resistant.

At 24 hours contact, their viability was 99.12-79.22% and 99.0-56.9%, respectively. Increased exposure up to 72 hours reliably caused a decrease in the survival of these cell lines – 96.24-68.1% and 82.2-51.7%. The loss of the solvent didn't affect the cytotoxic effect of the studied glues.

3. Conformation changes in albumin were manifested by a significant decrease in absorption in far UV region and at 278 nm wave length during its long-term contact with both "fresh" and "dried" glues. Manifestations of the toxic effect of glues on biomolecules were increased in the sequence: rubber > polyurethane > polychloroprene.

4. Shoe glues demonstrated an ability to generate free radicals in the sequence: rubber > polychloroprene > polyurethane. Pro-oxidant manifestations significantly were increased in a time period of 4 hours – 24 hours. That may create the risks related to their use.

5. The obtained results can be used to determine the targets and mechanisms of the toxic effect of shoe glues, to assessment of occupational exposure.

Contributors:

Lototska-Dudyk U.B. – project administration, conceptualization, data curation, writing – original draft, editing;

Kuzminov B.P. – project administration, conceptualization, validation, reviewing;

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