Suspending culture of erythrocytes in the assessment of the detergent functional component toxicity level

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**Background.** The study is devoted to the assessment of the cytotoxic properties of various surfactants by a method that is highly sensitive to identification of their negative effect on living mammalian cells.

**Objective.** The aim of the work is to carry out a comparative assessment of modern surfactants in terms of the degree of manifestation of cytotoxic properties.

**Methods.** The study of 10 surfactants was carried out with a new method for assessing the cytotoxic effect of agents based on surfactants, which is a modification of The RBC Hemolysis Test and Hemoglobin Denaturation Test. This method calculates the integral cytotoxicity index, which takes into account the data obtained by two tests. The following indicators were determined in the work: the percentage of hemoglobin denaturation in a 1% surfactant solution (D, %), the concentration of the surfactant solution at which hemolysis of 50% erythrocytes occurs (H50, %), and the cytotoxicity coefficient (C50, conv. un.) of surfactants which is calculated by the formula C50 = H50/D1000.

**Results.** According to the integral C50 indicator, the studied surfactants can be ranked according to the degree of toxicity reduction in the following order: sodium lauryl sulfate (0.09) — sodium laurate sulfate (0.13) — cocamidopropyl betaine (0.27) — sodium salt of polyethoxysulfosuccinate (0.27) — alkylidimethyl betaine (1.07) — disodium cocoamphodiacetate (1.99) — sodium salt n-palmethylglutamic acid (3.22) — cocoglucoside (27.86). Diethanolamides of coconut oil fatty acids and polyquaternium 7 in the studied concentration range (up to 1%) did not show denaturation properties.

**Conclusions.** Studies have shown that surfactants significantly differ from each other by the level of the damaging effect, the most aggressive components were anionic and amphoteric surfactants, nonionic surfactants have a significantly lower cytotoxic effect (10 times). The results obtained should be taken into account when creating soft agents based on surfactants.

**Keywords:** denaturation of hemoglobin; hemolysis of erythrocytes; anionic surfactants; amphoteric surfactants; nonionic surfactants; toxicity; damaging action.

Introduction

The active use of various surfactants in the household chemicals and cosmetics, as well as the development of new original surfactants with different specific and structure-forming properties, necessitate a preclinical assessment of their toxicological profile [1, 2]. Most often, when using surfactants, the calculation of the risk of toxic manifestations is based on the analysis of their chemical structure and the results of the experimental studies of their known analogues. Therefore, most of the available data on the safety of the surfactants are only predictions that cannot be used to compare their toxicological characteristics. Recent scientific publications indicate a prolonged dangerous effect of well-known surfactants of the detergents. It has been also experimentally demonstrated that in multicomponent formulations, these ingredients manifest the properties of enhancers that easily overcome the transdermal barrier. They penetrate through the horny layer into the deeper layers of the skin, interact with the proteins, changing the functional cells of the skin, increase the fluidity of lipid structures [3, 4]. The scientists have established that molecules of sodium lauryl sulfate penetrate to the epidermis, take root in its lipids, disrupt their synthesis and affect keratinocytes and corneocytes, resulting in increased transdermal water loss and perfusion of the epidermis, changed hydration of the horny layer and blood flow that cause the disorders of the skin barrier function [3—5]. That is, the surfactants are aggressive components of the detergents, and a degree of the manifestation of their damaging effects may be different. Therefore, it is relevant to conduct a comparative analysis of the cytotoxic properties of various surfactants by the methods that are the most sensitive ones to the detection of their negative effect on live mammalian cells.

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The goal of our work was to conduct a comparative assessment of modern surfactants by the degree of the manifestation of their toxic properties by the Method for the Assessment of Cytotoxic Effect of the Agents Based on Surfactants.

Materials and methods

Materials. Anionic surfactants: sodium lauryl sulfate, sodium laurate sulfate, sodium salt of polyethoxysulfosuccinate; amphoteric surfactants: disodium cocoamphodiacetate, alkyldimethylbetaine, cocamidopropylbetaine, sodium salt of n-palmitylglutamic acid; nonionic surfactants: cocogluicoside, diethanol amides of fatty acids of coconut oil.

Subjects. The short-term culture of guinea pig erythrocyte cells was prepared by technique [6].

Methods. The study was conducted according to our Method for the Assessment of the Cytotoxic Effect of the Agents Based on Surfactants, described in patent No. 28727 and scientific paper [6, 7]. This method is a modification of the RBC Hemolysis Test and Haemoglobin Denaturation Test [6]. The authors of the method, applied in the research, propose to use a culture of short-term erythrocyte suspension as an in vitro test system for toxicity. This methodological approach includes the assessment of the hemolytic effect of the agent and the percentage of denaturation of oxyhemoglobin in the mammalian erythrocyte culture, i.e. makes possible to evaluate the effect of the surfactants on the integrity of the erythrocyte cytoplasmic membrane (by the percentage of hemolysis of erythrocytes) and cellular proteins (by the percentage of denaturation of oxyhemoglobin) in the experimental solution. Hemolysis and denaturation characterize different processes of the manifestation of irritating effect of the agents that can complement each other. Therefore, the two methods’ study is performed in parallel and, when calculating the potential of cytotoxic action, takes into account the coefficient of cytotoxicity ($C_c$).

Evaluation of the effect of the surfactants on the integrity of hemoglobin (percentage of hemoglobin denaturation) was performed in several steps.

The first step: performance of 3 experiments in parallel: I experiment – cultivation of erythrocytes with the studied surfactant; II experiment – cultivation of the erythrocytes with a substance with proven toxicity (positive control); III experiment – cultivation of the erythrocytes with water (negative control), followed by the determination of the fugat optical density of their culture fluid at the wavelengths of 540 and 575 nm. The second step is to calculate the hemoglobin D denaturation coefficient according to the recommendations [6, 7].

The study of hemolytic properties of the surfactants was performed according to the method [6, 7]. Then we calculated the integral index – the cytotoxicity coefficient ($C_c$), which took into account the results of the experiments conducted by two methods. This integral index of cytotoxicity $C_c$ is calculated by the formula $C_c = \frac{H_{50}}{D \cdot 10000}$ where $C_c$ is the index of cytotoxicity (conventional unit); $H_{50}$ is the index of erythrocytes’ hemolysis (%); $D$ is the percentage of hemoglobin denaturation (%) [6, 7].

The studies carried out by this method have some limitations: they can not be used in the evaluation of dyed substances, insoluble ingredients, strong acids [7, 8]. If necessary, the proposed method can be used as an alternative one to the traditional methods for the determination of the skin irritation and effect on the mucous membrane of the eye, and as a method for the assessment of damaging effect level in the study of toxicity of the surfactants and agents based on them.

The obtained results were processed with the help of the traditional methods of variable statistics using the licensed computer programs Microsoft Excel and Statistica 10 [9].

Results and discussion

A surfactant is a multifunctional ingredient that provides, first of all, the effective action of synthetic detergents, dishwashing detergents, shampoos, shower gels, bath foams, liquid soaps, etc. In addition, the surfactants perform the structure-forming, emulsifying and thickening functions in the composition of household chemicals and cosmetics and are the enhancers for the biologically active additives. That is, the surfactants are practically an indispensable component for many cosmetic products and household chemicals, so the determination of the level of their toxicity is important for the creation of the formulations with a predicted lower level of damaging effect. Thus, a toxic effect of the surfactants on live cells is a prior indicator in the research program of the surfactant profiles.

For the comparative studies of cytotoxic effect, the surfactants were selected from the groups different by chemical structure and functional purpose. All of them are sold on the market in the form of stabilized aqueous solutions, their full composition and functional purpose are presented in Table 1.

The results of the study of hemoglobin denaturation process are presented in Table 2.
Table 1: Composition and functional purpose of stabilized solutions of surfactants chosen for study

| Surfactant                                      | Composition of stabilized solutions of surfactants | Functional purpose of surfactants [10]          |
|------------------------------------------------|--------------------------------------------------|-----------------------------------------------|
| Sodium n-dodecyl sulfate                        | 100% sodium lauryl sulfate                       | Main cleansing component, foaming agent       |
| Sodium laurate sulfate                          | 68–70% aqueous solution of sodium laurate sulfate| Main cleansing component, foaming agent       |
| Sodium polyethoxysulfosuccinate sodium          | 39–41% aqueous solution of sodium salt of polyethoxysulfosuccinate | Main cleansing component, foaming agent       |
| Cocoamidopropylbetaine                         | 30% cocamidopropylbetaine                       | Main and auxiliary cleansing component, foaming agent |
| Disodium cocoamphodyacetate                    | 37.5–39.5% aqueous solution of disodium cocoamphodyacetate, 11–12% sodium chloride | Main and auxiliary cleansing component, foaming agent |
| Alkyl dimethylbetaine                          | 30% aqueous solution of alkyl dimethylbetaine    | Main and auxiliary cleansing component, foaming agent |
| Sodium salt of n-palmityl glutamic acid         | 25–30.5% sodium salt of n-palmityl glutamic acid, 4–6% sodium chloride | Main and auxiliary cleansing component, foaming agent |
| Cocoglucoside                                  | 51–53% aqueous solution of cocoglucoside        | Cleansing component, foaming agent            |
| Diethanol amides of fatty acids of coconut oil | 100% diethanolamides of fatty acids of coconut oil | Viscosity modifier and foaming agent          |
| Polyquaternium 7                                | 40% aqueous solution of polyquaternium 7        | Conditioning additive                         |

Table 2: Percentage of hemoglobin denaturation in erythrocyte culture *in vitro* under the influence of surfactants

| Surfactant                                      | Percentage of hemoglobin denaturation $D$ (%) |
|------------------------------------------------|----------------------------------------------|
| Anionic surfactants                             |                                              |
| Sodium lauryl sulfate                           | 100.00 ± 0.50                               |
| Sodium salt of polyethoxysulfosuccinate         | 94.25 ± 0.58                                |
| Sodium laurate sulfate                          | 93.00 ± 0.88                                |
| Amphoteric surfactant                           |                                              |
| Sodium salt of n-palmityl glutamic acid         | 73.81 ± 0.37                                |
| Cocamidopropylbetaine                          | 37.00 ± 0.29                                |
| Alkyl dimethylbetaine                          | 28.00 ± 0.35                                |
| Disodium cocoamphodyacetate                    | 11.9 ± 0.18                                 |
| Nonionic surfactants                            |                                              |
| Cocoglucoside                                  | 2.38 ± 0.29                                 |
| Diethanol amides of fatty acids of coconut oil |                                              |
| Cationic surfactants                            |                                              |
| Polyquaternium 7                                |                                              |

The data above show that studied surfactants demonstrated a different ability to hemoglobin denaturation ($D$) at a preset standard concentration of the solution. Thus, by the reduction of the degree of destructive power of the surfactants per hemoglobin molecule (percentage of hemoglobin denaturation), they can be placed in the following order: sodium lauryl sulfate (positive control) – sodium salt of polyethoxysulfosuccinate – sodium laurate sulfate – sodium salt of n-palmitic glutamic acid – cocamidopropylbetaine – alkyl dimethylbetaine – disodium cocoamphodiacetate – cocoglucoside. Others (diethanol amides of coconut oil fatty acids, polyquaternium 7) did not show their protein-destroying properties in the standard concentration chosen for the study. That is, according to this method, the most aggressive surfactants are anionic ones, because all studied anionic surfactants (sodium lauryl sulfate, sodium laurate sulfate, sodium salt of polyethoxysulfosuccinate) demonstrated a high denaturation ability from 93 to 100%; amphoteric surfactants were less toxic ones – from 11.9 to 73.81%; nonionic surfactants were “the softest” ones – without effect or up to 2.38%; and cationic surfactants – polyquaternium 7 – without effect.
The study of hemolytic properties of the surfactants by means of the detection of the concentration of their solutions, when hemolysis of 50% of erythrocytes took place, was performed in the range of concentrations from 0 to 0.1% that was limited by the method of determination. The obtained results are presented in Table 3.

The data presented in Table 3 show that the only polyquaternium did not have hemolytic activity in a given range of concentrations and it did not show a denaturation ability when adding into the culture of erythrocytes. Others can be placed by the degree of H$_{50}$ increase, i.e. by the reduction of the toxicity, in such a way: sodium lauryl sulfate — cocamidopropylbetaine — sodium laurate sulfate — disodium cocoamphodiacetate — alkyl dimethylbetaine — cocoglusoside — sodium salt of polyethoxysulfosuccinate — sodium salt of n-palmityl glutamic acid — diethanol amides of fatty acids of coconut oil.

The obtained data of the studies of cytotoxic effect of the surfactants by two methods differ slightly in the degree of toxicity. Thus, high hemolytic activity was demonstrated by both anionic surfactants (sodium lauryl sulfate, sodium laurate sulfate) and amphoteric surfactants (cocamidopropylbetaine and disodium cocoamphodiacetate, alkyl dimethylbetaine), and when assessing the denaturation ability, the denaturation coefficient of cocamidopropylbetaine ($D - 37\%$), alkyl dimethylbetaine ($D - 28\%$) and disodium cocoamphodiacetate ($D - 11.9\%$) was significantly smaller than of sodium salt of polyethoxysuccinate ($D - 94.25\%$) and sodium salt of n-palmityl glutamic acid ($D - 73.81\%$). At the same time, by the hemolytic activity, the sodium salt of polyethoxysulfosuccinate ($0.007343\%$) and the sodium salt of n-palmityl glutamic acid ($0.023772\%$) were "softer" than disodium cocoamphodiacetate ($H_{50} = 0.002372\%$), alkyl dimethylbetaine ($H_{50} = 0.002989\%$), cocamidopropylbetaine ($H_{50} = 0.000996\%$) and cocoglusoside ($H_{50} = 0.00863\%$). The analysis of study results shows that there is no always clear inverse relationship between the hemolysis rate of erythrocytes and the rate of denaturation ability (higher hemolysis rate — lower denaturation rate), it means that the effect on protein structures and cell membrane in different surfactants may take place by different mechanisms of the effect depending on the chemical structure of substances [7, 8].

Therefore, the integrated cytotoxicity index $C_i$, which determines the degree of cytotoxic effect of surfactants, takes into account the results of two experiments.

At the absence of the process of hemoglobin denaturation in a preset surfactant solution, the coefficient $C_i$ is not calculated and only the hemolytic activity of the agents is evaluated. The findings, calculated by the formula above, are presented in Table 4.

According to the integral index $C_i$, the examined surfactants can be placed by the degree of reduction of toxicity in the following order: sodium lauryl sulfate — sodium laurate sulfate — cocamidopropylbetaine — sodium salt of polyethoxysulfosuccinate — alkyl dimethyl betaine — disodium cocoamphodiacetate — sodium salt of n-palmityl glutamic acid — cocoglusoside — diethanolamides of fatty acids of coconut oil — polyquaternium 7.

| Surfactant | $(\%)$, takes place |
|------------|---------------------|
| Sodium lauryl sulfate | 0.00863 ± 0.0000049 |
| Sodium laurate sulfate | 0.001123 ± 0.0000009 |
| Sodium salt of polyethoxysulfosuccinate | 0.007343 ± 0.0000123 |
| Cocamidopropylbetaine | 0.000996 ± 0.0000006 |
| Disodium cocoamphodiacetate | 0.002372 ± 0.0000009 |
| Alkyl dimethylbetaine | 0.002989 ± 0.0000024 |
| Sodium salt of n-palmityl glutamic acid | 0.023770 ± 0.0000058 |
| Cocoglusoside | 0.006631 ± 0.0000027 |
| Diethanol amides of fatty acids of coconut oil | 0.058888 ± 0.0000012 |
| Polyquaternium 7 | – |
Table 4: Values of surfactant cytotoxicity integral index

| Surfactant                                      | Integral index of surfactant cytotoxicity $C_c$ (conv. un.) |
|------------------------------------------------|---------------------------------------------------------------|
| **Anionic surfactants**                        |                                                                |
| Sodium lauryl sulfate                           | 0.09 ± 0.0006                                                  |
| Sodium laurate sulfate                          | 0.13 ± 0.0009                                                  |
| Sodium salt of polyethoxysulfosuccinate         | 0.78 ± 0.0012                                                  |
| **Amphoteric surfactant**                      |                                                                |
| Cocamidopropylbetaine                          | 0.27 ± 0.0015                                                  |
| Disodium cocoamphodiacetate                     | 1.99 ± 0.0019                                                  |
| Alkyldimethylbetaine                            | 1.07 ± 0.0023                                                  |
| Sodium salt of n-palmitylglutamic acid          | 3.22 ± 0.0002                                                  |
| **Nonionic surfactants**                       |                                                                |
| Cocoglucoside                                   | 27.6 ± 0.0012                                                  |
| Diethanol amides of fatty acids of coconut oil  | –                                                              |
| **Cationic surfactants**                       |                                                                |
| Polyquaternium 7                                | –                                                              |

**Conclusions**

Analysis and generalization of study results, conducted by the Method of the Assessment of the Cytotoxic Effect of the Agents Based on the Surfactants, show that this method makes it possible to detect the degree of cytotoxic effect of the surfactants. The anionic and amphoteric surfactants were the most aggressive components and nonionic surfactants had a much lower cytotoxic effect (10 times less).

Taking into account a different degree of toxicity of studied substances, the manufacturers of cleansings that seek to create the "soft" special-purpose products, for example, such as cleansings for children’s skin and hair, intimate hygiene, washing of children's dishes, may be recommended to introduce less cytotoxic substances in terms of $C_c$ into the formulations or to reduce the percentage of anionic surfactants (or remove them) by increasing nonionic and "soft" amphoteric surfactants.

In the future, we plan to develop a comprehensive program for the study of the safety of surfactants using the Method for the Assessment of the Cytotoxic Effect of the Agents Based on Surfactants at the screening stage.

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СУСПЕНЗІЙНА КУЛЬТУРА ЕРИТРОЦІТІВ У ОЦІНКІ СТУПЕНЯ ТОКСИЧНОСТІ ФУНКЦІОНАЛЬНИХ КОМПОНЕНТІВ МИЙНИХ ТА ОЧИЩАЮЧИХ ЗАСОБІВ

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Проспільнування, присвячене оцінці цитотоксичних властивостей різних поверхнево-активних речовин (ПАВ) метою, який є високочутливим до виявлення їх негативного впливу на живі клітини ссавців.

Мета. Провести порівняльну оцінку сучасних ПАВ за ступенем виявленості цитотоксичних властивостей.

Методика реалізації. Проведені дослідження показали, що за рівнем пошкоджуючої дії сурфактанти значно різняться між собою, на відтак декілька компонентів виявились аніонні, амфотерні ПАВ, а значно меншу цитотоксичну дію (в 10 разів) мають неіонні ПАВ.

Результати. Проведені дослідження показали, що за рівнем пошкоджуючої дії сурфактанти значно різняться між собою, на відтак декілька компонентів виявились аніонні, амфотерні ПАВ, а значно меншу цитотоксичну дію (в 10 разів) мають неіонні ПАВ.

Висновки. Проведені дослідження показали, що за рівнем пошкоджуючої дії сурфактанти значно різняться між собою, на відтак декілька компонентів виявились аніонні, амфотерні ПАВ, а значно меншу цитотоксичну дію (в 10 разів) мають неіонні ПАВ.