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Original Article

ASSESSMENT OF BIOLOGY ACTIVITY OF THE PEELING SUBSTANCES BY THE PHYSICOCHEMICAL APPROACHES ON THE *SPIROSTOMUM AMBIGUUM* CELL MODEL

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ABSTRACT

Objective: To evaluate the biological activity of chemical peeling substances based on enzymatic and Arrhenius kinetics using *Spirostomum ambiguum* as an alternative approach to animal experiments.

Methods: The *Spirotox* method was used to analyze the mechanism of «xenobiotic-cell» interaction, similar to the Michaelis-Menten enzymatic kinetics. The Hill-Langmuir equation was used to determine the degree of cooperativity in the binding of xenobiotics to cellular receptors. Using the Arrhenius kinetics, the observed activation energy obsEa of cell death in the model solutions of glycolic and carbolic acids was determed, which will allow predicting the toxicity parameters of any peeling substances.

Results: The relationship Spirostomum ambiguum lifetime t_L-lgC concentration of peeling compound solution made it possible to characterize the moment of cellular transition from the intermediate state $C{\bullet}L_n$ to the dead state DC, characterized by irreversible structural and functional changes in the cell/death. The values were 5.3 mmol•l^{-1} for glycolic acid solutions and 2.8 mmol•l^{-1} for carbolic acid solutions. Equilibrium constants K_{eq} of complexation, the rate of infusoria death f_m, and the degree of ligand cooperativity n were calculated. The activation energy $^{obs}E_a$ of cell death was determined in Arrhenius coordinates, which were 210±0.39 kJ·mol^{-1} and 108±0.09 kJ·mol^{-1} for glycolic and carbolic acids respectively. The correlation between the values of activation energy and DL_{50} of mammals (rats) was discovered.

Conclusion: The obtained kinetic parameters made it possible, without animals and humans testing, to characterize the mechanisms of interaction of peeling substances with the living cell.

Keywords: Peeling, Glycolic acid, Phenol, *Spirotox* method, Biological activity, Animal-free test

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INTRODUCTION

Chemical peeling (chemexfoliation) stimulates the appearance of controlled keratocoagulation and denaturing proteins in the epidermis and dermis, the release of pro-inflammatory cytokines and chemokines, and the production of new skin collagen and elastin [1-5]. The clinical effectiveness of chemical peeling, as well as the risk of various complications, depends on many factors: the properties of chemicals, concentration, regimen, and the number of uses, skin type, dermatological condition, and cumulative dose [6, 7]. Despite available reviews about the traumatic nature of this procedure, chemical peeling ranks third in the frequency of non-invasive cosmetic procedures in the USA [8]. Since the use of cosmetic products is not associated with an urgent need for the life and health of consumers, there is the number of advocates of alternative *in vitro* approaches to animal experiments, for example, when checking the safety of cosmetics [9-14]. Cosmetic animal testing is banned in a few countries [15]. For the testing of cosmetics, programs are being developed to enable the cosmetics industry to conduct safety assessments [16, 17]. To date, testing methods without the use of animals have been selected, including cell lines [18].

Alternative models use various cellular cultures, specialized moving cells, bacteria, single-celled organisms, primitive crustaceans and other hydrobionts. Methods based on the reactions of the simplest animals are of paramount importance in today's environment, as they allow determining the possible range of properties in a short period the substance under study and making recommendations on whether to investigate it further. For example, there are approaches to describing the kinetics of the development of cellular population under the influence of inhibitors and promoters [19]. It is also important that biological research methods using protozoa significantly reduce the time required to study the properties of substances and are characterized by a relatively low cost. Replacing warm-blooded animals with unicellular ones reduces the time of toxicological and pharmaceutical studies from one year to a month, and the cost of their implementation decreases in ten times [20]. The goal is to directly replace animal testing with non-animal methods. Advances in cell and molecular biology and informatics need to be leveraged to develop new specific preclinical testing strategies that are applicable to specific human situations [21].

This work aims to evaluate the biological activity of chemical peeling substances using enzymatic and Arrhenius kinetics for a single-cell model *Spirostomum ambiguum* as an alternative approach to animal experiments.

MATERIALS AND METHODS

Cell model

The test culture *Spirostomum ambiguum* (*Sp. ambiguum)* has been cultured in the laboratory to carry out studies of individual and combined biological activity of medicines.

The protozoan ciliate *Sp. ambiguum* is characterized by tape-shaped, dorsal body shape (1-3 mm long), has a macronucleus clear-shaped and mouth apparatus up to the back third of the body (fig. 1).

Compared to other objects of biological testing, *Sp. ambiguum* have several advantages, since they are eukaryotic organisms. The statistically reliable sensitivity to toxicants makes it possible to compare the response of protozoa with that of humans [23, 24].

Under favorable conditions in a low-mineralized environment, cells do not die for a period exceeding their cell cycle (about 20 h).

When incubated into a toxicant solution, cells die over time, which is a function of both concentration and temperature. Kinetic scheme of ligand-induced death of *Sp. ambiguum* includes the reversible reaction with the formation of an intermediate product ($C \bullet L_n$) and the state of transition to cell death (DC) (fig. 2).

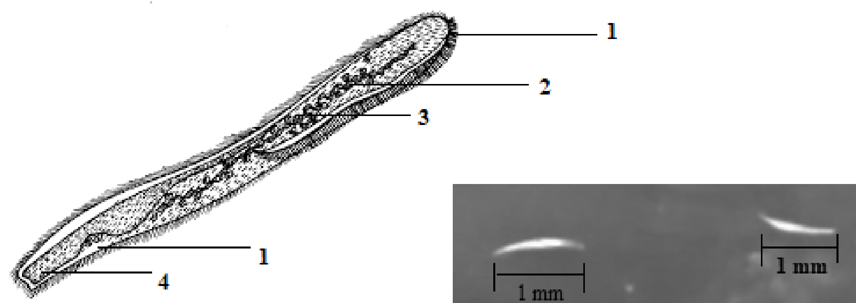


Fig. 1: The general features of *Sp. ambiguum* ciliates: 1–peristom; 2–large macronucleus; 3–small micronuclei; 4–reducing vacuole; in the insert–a light microscope image of *Spirostomum ambiguum* in real laboratory settings [22]

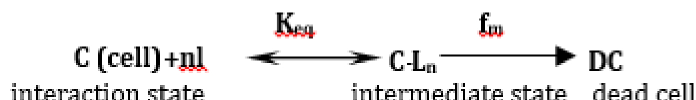
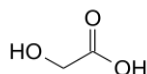


Fig. 2: Kinetic scheme of ligand-receptor interaction *Sp. ambiguum* with xenobiotic (L) [25]

The presence of an intermediate state of ligand-induced death means that the process of cell death should be activated and proceeds with energy consumption [26, 27]. Indeed, we have established the dependence of *S. ambiguum* mortality on temperature using the models of pharmaceutical substances for chemical peeling.

Powder substance samples

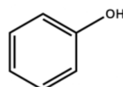
1. Glycolic acid ($HOCH_2COOH$), 2-Hydroxyacetic Acid (fig. 3) – colorless hygroscopic crystals, that soften, separate, and cause desquamation of the cornified epithelium or horny layer of skin (ReagentPlus®, 99% Sigma-Aldrich) [28].



2-Hydroxyethanoic acid

Fig. 3: Chemical structure of glycolic acid

2. Carbolic acid (C_6H_6O) also known as phenol, hydroxybenzene (fig. 4). It is also used as an antiseptic and disinfectant (chemical that kills bacteria and fungi) and in medicinal preparations such as mouthwash and sore throat lozenges (BioUltra, for molecular biology, ≥99.5% Sigma-Aldrich).



Benzenol

Fig. 4: Chemical structure of carbolic acid

Technique of research

The installation of the *Spirotox* method includes the following blocks: a glass thermostatic cell with five recesses for solutions; Lauda AlphaA 6 thermostat; Binoculars IBS-10; Daylight lamp (10 W) for field observations. The analyzed sample aliquot and 3-5 test culture individuals were introduced into the thermostatic cell holes (V = 150µl). The pipette with a diameter of more than 1 mm was used to avoid infusoria injuries. The death of the cell was stated by the rupture of the membrane with the release of the contents of the protoplasm outwards or by immobilization with no contractile reaction to mechanical irritation.

Statistics

The findings were processed by the statistical methods using software packages of Origin Pro 9.1. Each value on the fig. represents as average mean±SD; p values less than 0.001 were considered as significant.

RESULTS AND DISCUSSION

Determination of chemical peelings solution toxic concentration

To study the biological activity of glycolic acid on the cell model the range of six concentrations values from 4.5 to 12.0 $mmol \cdot l^{-1}$was chosen to visually monitor the behavior of ciliates. For carbolic acid solutions, the test range of concentrations ranged from 2.0 to 6.1 $mmol \cdot l^{-1}$(fig. 5). The graphs in semilogarithmic coordinates made it possible to determine the boundary values of the concentration of the cell transition from the intermediate state $C \bullet L_n$ to the dead state DC. The intermediate state is characterized by a change in the morphometric characteristics of cells, the appearance of cytoplasmic grains and a decrease in the intensity of movement. The rupture of the cell membrane and the release of endoplasm into the external environment were accompanied by cell death at a concentration of glycolic acid solutions above 5.3 $mmol \cdot l^{-1}$and carbolic acid above 2.8 $mmol \cdot l^{-1}$.

Determination of the affinity for the cell receptor degree

Using the idea of a living cell as a multi-fermented multiphase chemical reactor [29-31] allows to describe the interaction of *Sp. ambiguum* receptors with chemical peels substances by the Hill equation (at n = 1 we obtain the Michaelis-Menten equation):

$$^{obs}f_m = f_m(1+K_{eq}/[L]^n) \text{ .... (1)}$$

The expression for *Sp. ambiguum* lifetime (t_L) can be written in the form [32-34]:

$$t_L = 1/f_m + K_{eq}/f_m \times 1/[L]^n, \text{ .... (2)}$$

Where $^{obs}f_m$ is the observed rate constant of the cell transition to the dead state (cell death rate), K_{eq} represents the equilibrium constant, [L] is the xenobiotic concentration and *n*-stoichiometric coefficient in kinetic scheme (fig. 2).

The straight line of the dependence $t_L = F(1/[L]^n)$ makes it possible to determine the values of the death rate $f_m = 1/t_L$ at the ordinate intersection and the equilibrium constant of the formation of the intermediate complex K_{eq} at the abscissa intersection with an appropriate choice of stoichiometric coefficient *n* [32-36]. Calculations have shown that for glycolic acid n = 2 and for carbolic acid n = 3. The linearization of the experimental results in the indicated coordinates at other values is less reliable (fig. 6, table 1). Thus, the using kinetic model describes the mechanisms of ligand-receptor interaction of the xenobiotics with the living cell.

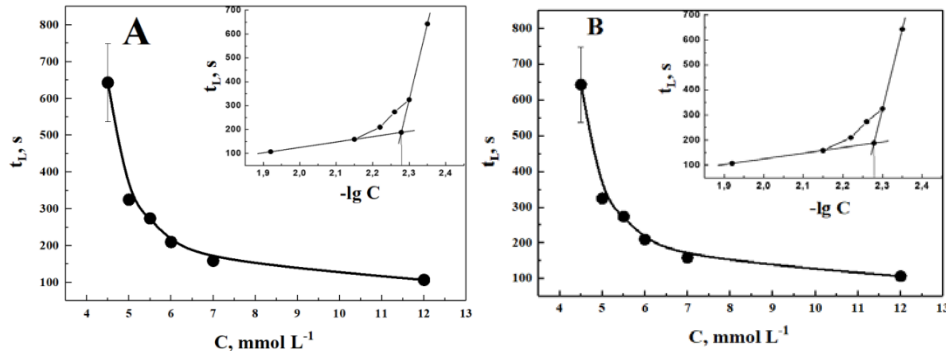


Fig. 5: Dependence of cellular biosensor lifetime on the concentration of water solutions of glycolic (A) and carbolic (B) acids; n=5; p<0.001. The inset shows the dependence t_L=f (lgC)

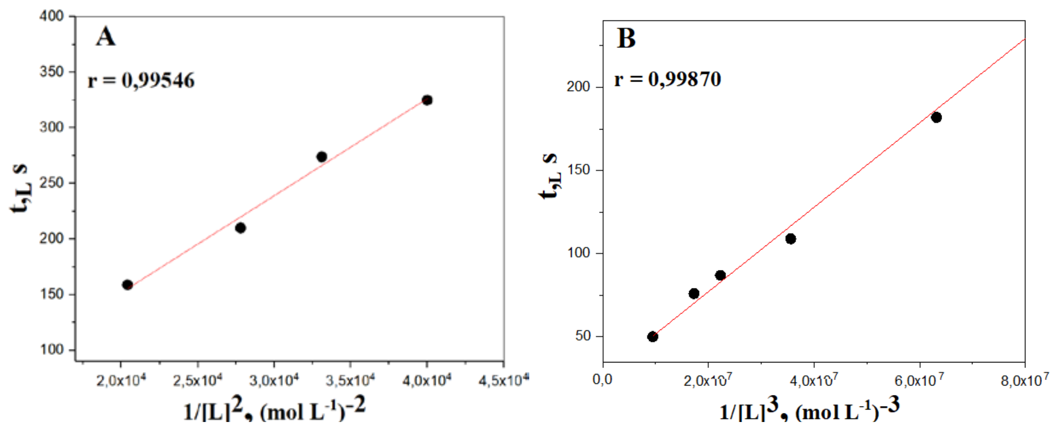


Fig. 6: Relationship of the lifetime of *Sp. ambiguum* as a function of the concentration of glycolic (A) and carbolic (B) acids

Table 1: Kinetic parameters of ligand-receptor interactions of the chemical peeling agents and *Sp. ambiguum* (n= 5; p<0.001)

Chemical peeling agent	Toxic concentration, *C*, mmol·l⁻¹	Stoichiometric coefficient, *n*	Equilibrium constant of intermediate state, K_{eq}	The rate constant of a cell transition to the DC state, f_m, s⁻¹
Glycolic acid	5.3	2	$7.50 \cdot 10^{-5}$	$9.40 \cdot 10^{-3}$
Carbolic acid	2.8	3	$1.14 \cdot 10^{-7}$	$4.42 \cdot 10^{-2}$

The K_{eq} and f_m values reflecting the mechanism of the toxic effect of aqueous solutions for chemical peeling at the stage of the formation of the intermediate complex and the reaction product are analogous to the Michaelis-Menten constant (K_m) and V_{max} at *n* = 1.

Thus, the efficiency of the ligand-receptor interaction in the "chemical peeling agent-living cell" system can be assessed using the Spirotox eukaryotic cell model. Low values of the equilibrium constant K_{eq} for the formation of the intermediate product $C \bullet L_n$, as well as high values of the rate f_m of the cell's transition to the lethal state with an increase in the number of attached ligands *n*, indicate a high biological activity of the xenobiotic. This is clearly demonstrated by both glycolic and carbolic acids and by other xenobiotics studied earlier [37, 38].

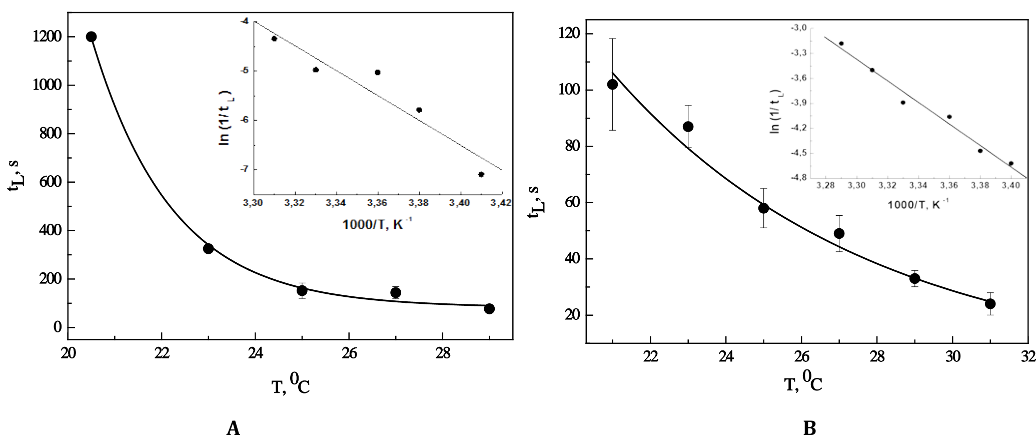


Fig. 7: Relationship of the lifetime of *Sp. ambiguum* as a function of temperature in direct and Arrhenius coordinates at infusoria incubation in solutions of glycolic (5 mmol·l⁻¹)–A and carbolic (3 mmol·l⁻¹)–B acids; n=5; p<0.001

Temperature dependence of the rate of *Sp. ambiguum* death in solutions of peeling acids

The presence of an intermediate state C • Ln in the process of ligand-induced death of the test object means that the process of cell death must occur with the consumption of energy. Using the examples of xenobiotics of different natures, we found that ligand-induced cellular transformations are linearized in Arrenius coordinates:

$$\ln k = \ln A - \frac{E_a}{R} \cdot \frac{1}{T} \ldots (3)$$

here k is the rate constant, A is the pre-exponential factor, E_a is the activation energy ($J \cdot mol^{-1}$), R is the ideal gas constant ($8.314 J \cdot mol^{-1} \cdot K^{-1}$), and T is the absolute temperature.

In semilogarithmic coordinates, the tangent of the straight line slope $\ln(1/t_L) = F(1/T)$ to the abscissa axis is E_a/R (fig. 7) [39, 40].

The values of observed activation energy (^{obs}Ea) for the test compounds have been found, using Arrhenius coordinates (table 2).

Table 2: The calculated ^{obs}Ea values of ligand-induced *S. ambiguum* death process in water solutions of the chemical peeling agents; n=5; p<0.001

Chemical peeling agent	$^{obs}Ea \pm SD$, $kJ \cdot mol^{-1}$
Glycolic acid	210±0.39
Carbolic acid	108±0.09

The lower activation energy, as well as the reduction of *Sp. ambiguum* lifetime by about ten times at the same temperature for carbolic acid compared to glycolic acid, indicates higher biological activity of this pilling agent. According to our data, substances with high biological activity, characterized by low cell survival, correspond to low ^{obs}Ea values, which are consistent with the values toxicity for organisms of a higher hierarchical level (fig. 8).

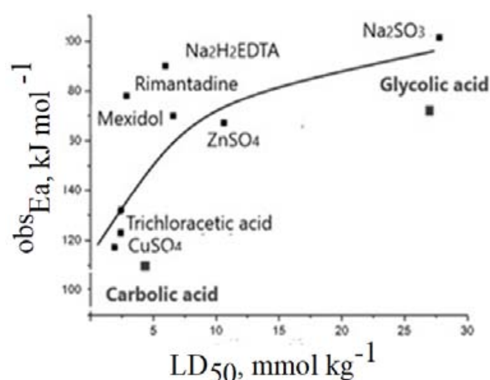


Fig. 8: Dependence of the $^{obs}E_a$ of *Sp. ambiguum* mortality on average lethal doses of LD_{50} ($mmol \cdot kg^{-1}$, rats, *per os*) for different pharmaceutical substances

As would be expected, there is a high correlation between the values of the apparent activation energy and LD_{50} (rats, *per os*) of chemical peeling substances and other pharmaceuticals [41].

CONCLUSION

Animal experiments can predict the effectiveness and safety of drugs in humans. Millions of mice, rats, hamsters, guinea pigs, rabbits, cats, dogs, monkeys and chickens are used for research, educational, and industrial purposes. Ethical issues with the use of experimental animals are the result of a conflict between the importance of animal experimentation to basic science and ethical principles that prevent actions from causing pain and suffering. The article presents the results of studies that exclude ethical conflicts. The efficiency of the ligand-receptor interaction in the "chemical peeling agent-living cell" system was assessed using the *Sp. ambiguum* model of eukaryotic cells. The *Spirotox* method was based on the Michaelis-Menten enzymatic kinetics and the temperature dependence of the Arrhenius reaction rate. Low values of the equilibrium constant K_{eq} for the formation of the intermediate product $C{\bullet}L_n$, high values of the rate f_m of cell transition to the lethal state and the degree of cooperativity n indicate a high biological activity of the xenobiotic. The correlation between the values of activation energies $^{obs}E_a$ and DL_{50} of mammals was proved.

The developed physicochemical and biological models can be used for any peeling substances.

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AUTHORS CONTRIBUTIONS

PTV developed the concept of the study. UEV designed the study and guided and supervised the study. PTV and UEV contributed to the interpretation of data and wrote the first draft. PMH and KIV was associated in supervising, advising and positioning the manuscript. All authors read and made corrections to the finalized manuscript before submission.

CONFLICTS OF INTERESTS

The authors declare that there is no conflict of interests.

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