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THE FLUORESCENT PROBE METHOD IN INVESTIGATION OF THE STATE OF ERYTHROCYTE MEMBRANES IN WHITE RATS AT EXPOSURE TO CHEMICAL ENVIRONMENTAL FACTORS

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The purpose of the presented work was to study the possibility of using the fluorescent probes method to diagnose the harmful effects of chemical factors on the example of polyethylene glycol on the organism of white rats by evaluating the state of erythrocyte membranes.

Material and methods. We used the following fluorescent probes in the studies: ortho-hydroxy derivatives of 2.5-diaryl-1.3-oxazole.

Results and discussion. In the case of erythrocytes of rats, which are toxic to PEG-400, there is a marked decrease in the fluorescence intensity of all the probes used. The discussed decrease in the fluorescence intensity of the probes indicates a decrease in the number of molecules of each of the probes associated with erythrocyte membranes per one hour of incubation, which indicates a decrease in the rate of binding of probes to membranes. Such a decrease in the binding rate can be explained by the formation of an additional protective membrane around each lipid membrane.

Conclusion. The established fact of formation of an additional coat of polyethylene glycol molecules on the surface of erythrocyte membranes can be treated as a stable standardized indicator for the method of fluorescent probes, which may indicate the absence of damaging effect on the membranes at the object of study, which requires confirmation in further studies.

Keywords: chemical factors, polyethylene glycol, toxification, erythrocytes, biomembrane, lipid bilayer, fluorescent probe.

Research relation to the plans, programs and department themes. The research was carried out within the framework of performing the research work of Kharkiv National Medical University on the special order of the Ministry of Health of Ukraine "Experimental substantiation of the prognosis of danger and correction of structural and pathogenetic disorders in the body warm-blooded with the purpose of developing hygienic standards for surfactants for water in reservoirs" (state registration number 0115U000233), as well as the initiative research work of biological chemistry department "Biochemical mechanisms once dismetabolic processes under the influence of chemical environmental factors "(state registration number 0115U000240).

Introduction. The problem of diagnosing the consequences of harmful effects on the body of chemical environmental factors (CEF) is relevant, because today scientists of Ukraine and such countries as the United States of America, Great Britain, Germany and France are concerned about the growing pollution of drinking water and the world's oceans, and the entry of chemical pollutants into the human body with cosmetics, medicines, shampoos, detergents, materials for arranging apartments and many others [1-6]. Chemical factors have radiomimetic properties and cause a wide range of various dismetabolic disorders in the body. One of the most commonly used in the national economy and everyday life of a person is polyethylene glycol (PEG) [7].

PEG is used to soften some plastics (polyethylene, polyvinyl chloride, cellulose ether membranes in filters, etc.) that are used to store and transport food. According to the latest research of foreign scientists, plastic components can migrate from products made from them to foodstuffs during storage, processing and transportation, and thus potentially affect human health [1, 6].

The Purpose of the presented work was to study the possibility of using the fluorescent probes method

to diagnose the harmful effects of chemical factors on the example of polyethylene glycol on the organism of white rats by evaluating the state of erythrocyte membranes.

Material and Methods. The chemical factor polyethylene glycol-400 (PEG-400) produced by "Barva-Pharm", Ivano-Frankovsk, was used as an object of research. For the study, an active dose of 1/10 LD_{50} was chosen according to the indicators of general toxic effect on the warm-blooded organism [7].

Polyethylene glycol-400 (PEG-400) is a colorless viscous liquid with a characteristic odor and bitter, slightly burning taste, very hygroscopic. The empirical formula is $HOCH_2$ (CH_2OCH_2) mCH_2OH, where m is the average number of oxyethylene groups. PEG is obtained by polymerization of ethylene oxide in the presence of water and a catalyst under pressure [1].

A 45-day study was performed on 20 white rats of both sexes of the WAG line of the control and test groups in an amount of 10 animals each. Animals were in the standard conditions of the vivarium. The content and monitoring of animals was carried out in accordance with the provisions of the "General principles of animal experiments", agreed upon by the First National Congress on Bioethics (Kiev, 2001), "European Convention for the Protection of Vertebrates used for experimental and scientific purposes" (Strasbourg, 1986).

An aqueous solution of PEG-400 was daily injected intragastrically at a dose of 1/10 DL_{50} using a metal probe. The mean lethal dose (DL_{50}) for polyethylene glycol according to these parameters of acute toxicity was established at 28.9 g / kg body weight of white rats, 1/10 DL_{50} was 2.89 g / kg body weight [7]. The control group of rats received the corresponding volumes of drinking water. After the end of the 45-day subacute toxicological experiment, rats were withdrawn from it in accordance with the "International recommendations for conducting biomedical studies using laboratory animals" by decapitation using a guillotine, according to approved instructions and legislative acts.

Erythrocytes were separated from the plasma by centrifugation (UNIVERSAL 320 R centrifuge) with stabilized blood heparin (the final dilution of heparin-whole blood was 1: 100) for 15 min at 3000 g. The erythrocyte suspension was washed several times with a cooled 0.89% solution of NaCl [8].

Fluorescent probes - ortho-hydroxy derivatives of 2.5-diaryl-1.3-oxazole, molecules of which are noncovalently bind to cell membranes and react to changes in the microenvironment [9-12], which were previously successfully used for biomembrane studies: 2- (2'-OH-phenyl) -5-phenyl-1.3-oxazole (probe 010), 2- (2'-OH-phenyl) -5- (4'-phenyl-phenyl) -1.3-oxazole (probe 060) and 2- (2'-OH-phenyl) -phenanthrene (10,11) -1,3-oxazole (PH7 probe) [13-16].

The choice of fluorescent probes 010, 060 and PH7 (ortho-hydroxy derivatives of 2.5-diaryl-1.3-oxazole) for the study of erythrocyte membranes is due to the fact that the fluorescent characteristics of these probes depend on the physicochemical properties of their microenvironment: from the proton donor ability, polarity and viscosity of microenvironment [9–12].

The fluorescent probes were dissolved in acetonitrile to an initial concentration of 2 × 10 -4 mol / L, then 50 μl of each of the respective probe solutions were added to 2 ml of the red blood cell suspension. The final concentration of each of the probes in the suspension of the investigated membranes was 5·10-6 mol / l, so the molar ratio of lipid / probe was 200: 1. Fluorescence spectra were measured on a Lumina (Thermo Fisher Scientific) spectrophotometer one hour after the addition of probes to the erythrocyte suspension. Fluorescence spectra of probes were measured in the 340–600 nm regions with a width of the slits of excitation and fluorescence monochromators of 5 and 5 nm, respectively, and an excitation wave length of 330 nm. The error in measuring the fluorescence intensity was 5%.

Results and Discussion. Fluorescent probes-the ortho-hydroxy derivatives of 2.5-diaryl-1.3-oxazole, sensitive to changes in the proton-donor capacity, polarity, and viscosity of the microenvironment, were used to study the state of membranes of rat erythrocytes under the influence of PEG-400 [9–12]. Ortho-hydroxy derivatives of oxazole are distinguished, differing in their lipophilicity: it is expected that the regions of localization of the selected probes in the membrane are different and correspond to the lipophilicity of the probes (**Figure 1**) [13–16]. The expected localization and orientation of 010, 060 and PH7 on the basis of their fluorescent properties in lipid membranes [13-16] and on the basis of their structural similarity with fluorescent probes with known localization in lipid membranes [17]: probe 010 – in the region of glycerol residues of phospholipids (closer to the center of the lipid bilayer), in the region of carbonyl groups of phospholipids and in the region of fatty acid chains of phospholipids adjacent to the region of carbonyl groups; probe 060 – in the region of carbonyl groups of phospholipids and in the region of fatty acid chains of phospholipids (near the polar part of the bilayer); probe PH7 – in the region of fatty acid chains of phospholipids (near the center of the bilayer) and in the center of the lipid bilayer of membranes (**Figure 1**).

The results of measuring the fluorescence of probes in solutions containing rat erythrocytes under the influence of PEG-400 and erythrocytes of the control group are shown in **Figure 2**.

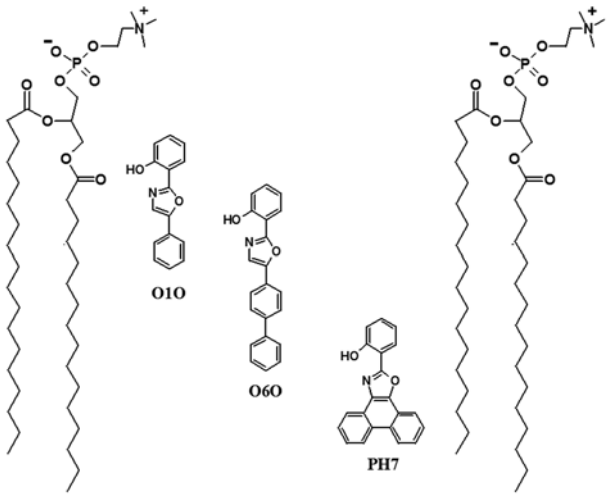

Fig. 1. Expected localization and orientation of fluorescent probes 010, 060 and PH7 based on their fluorescent properties in lipid membranes [14, 15] and on the basis of their structural similarity with fluorescent probes with known localization in lipid membranes [17]. To indicate the location of the probes, two molecules of phosphatidylcholine from an external monolayer

According to **Figure 2**, in the case of the animals of the experimental group, the ratio of the long-wavelength intensities (emission of the phototometric shape of the probe [9–12]) and the short-wave (emission of the normal shape of the probe [9–12]) fluorescence bands for each of the probes used was practically unchanged in comparison with the corresponding value for of the control group of rats: for probe 010, the fluorescence intensity ratio I477 / I370 was 110 and 108, respectively; for probe 060, the fluorescence intensity ratio I490 / I386 was 18 and 17, respectively, and, for the PH7 probe, the fluorescence intensity ratio I490 / I400 was 25 and 23, respectively. Thus, as a result of the influence of PEG-400, no changes are observed in the localization of the 010, 060, and PH7 probes, i.e. in the field of glycerol residues of phospholipids, in the region of carbonyl groups of phospholipids and in the region of fatty acid chains of phospholipids.

At the same time, in the case of erythrocytes of rats (**Figure 2**), which are toxic to PEG-400, there is a marked decrease in fluorescence intensity of all the probes used. The discussed decrease in the fluorescence intensity of the probes indicates a decrease in the number of molecules of each of the probes associated with erythrocyte membranes per one hour of incubation, which indicates a decrease in the rate of binding of probes to membranes. Such a decrease in the binding rate can be explained by the formation around each lipid membrane of an additional protective shell [18] consisting of polyethylene glycol molecules adsorbed on the membrane surface [19].

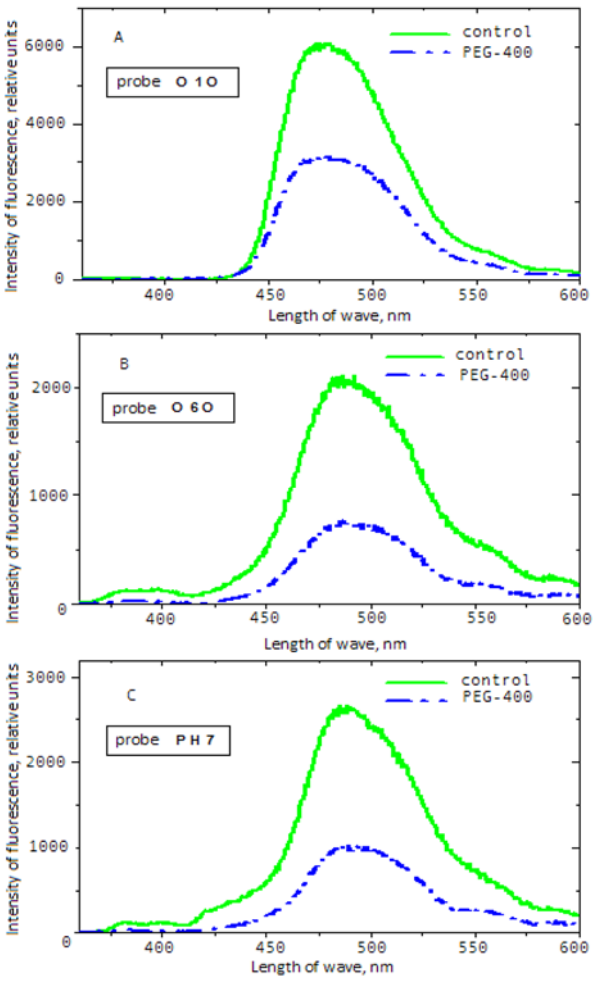

Fig. 2. Fluorescence spectra of probes 010 (A), 060 (B) and PH7 (C) in solutions containing rat erythrocytes: under the influence of PEG-400 (dashed line), intact (solid line)

Conclusions

1. The registered dynamics of fluorescence intensity of probes, as well as the absence of changes in the region of glycerol residues of phospholipids, in the region of carbonyl groups of phospholipids and in the region of fatty acid chains of erythrocyte phospholipids of the experimental group of animals treated with PEG-400 at a dose of 1/10 LD50, is evidence of no damage on the membrane.
2. The fact of forming an additional shell of polyethylene glycol molecules on the surface of membranes of blood erythrocytes of rats is established. The obtained results by the method of fluorescent probes can be treated as a stable standardized indicator, which can indicate the absence of damaging effect on the membranes of the object of study.

Prospects for further research. Further studies will deal with studying the types and stages of apoptosis of nucleated cells.

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ДОСЛІДЖЕННЯ МЕТОДОМ ФЛУОРЕСЦЕНТНИХ ЗОНДІВ СТАНУ МЕМБРАН ЕРИТРОЦИТІВ БІЛИХ ЩУРІВ ПРИ ВПЛИВІ ХІМІЧНИХ ФАКТОРІВ НАВКОЛИШНЬОГО СЕРЕДОВИЩА

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Метою представленої роботи було вивчення можливості застосування методу флуоресцентних зондів для діагностики шкідливого впливу хімічних факторів на прикладі поліетиленгліколю на організм білих щурів шляхом оцінки стану мембран еритроцитів.

Методи. У дослідженнях використані флуоресцентні зонди – орто-гідроксипохідні 2,5-діарил-1,3-оксазолу.

Результати. У випадку еритроцитів щурів, токсіфікованих ПЕГ-400, спостерігається помітне зниження інтенсивності флуоресценції всіх використаних нами зондів. Встановлене зниження інтенсивності флуоресценції зондів свідчить про зменшення кількості молекул кожного з зондів, що зв'язалися з мембранами еритроцитів за одну годину інкубації, що свідчить про зменшення швидкості зв'язування зондів з мембранами. Таке зменшення швидкості зв'язування може бути пояснено формуванням навколо кожної ліпідної мембрани додаткової захисної оболонки.

Висновок. Встановлений факт формування на поверхні мембран еритроцитів додаткової оболонки з молекул поліетиленгліколю методом флуоресцентних зондів можна трактувати як стабільний стандартизованность показник свідчить про відсутність шкідливої дії на мемрани у об'єкта вивчення, що вимагає підтвердження в подальших дослідженнях.

Ключові слова: хімічні фактори, поліетиленгліколь, токсифікація, еритроцити, біомембран, ліпідний бішар, флуоресцентний зонд.

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ИССЛЕДОВАНИЕ МЕТОДОМ ФЛУОРЕСЦЕНТНЫХ ЗОНДОВ СОСТОЯНИЯ МЕМБРАН ЭРИТРОЦИТОВ БЕЛЫХ КРЫС ПРИ ВОЗДЕЙСТВИИ ХИМИЧЕСКИХ ФАКТОРОВ ОКРУЖАЮЩЕЙ СРЕДЫ

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Резюме. *Целью* представленной работы явилось исследование возможности применения метода флуоресцентных зондов для диагностики вредного воздействия химических факторов на примере полиэтиленгликоля на организм белых крыс путём оценки состояния мембран эритроцитов.

Методы: В исследованиях использованы флуоресцентные зонды – орто-гидроксипроизводные 2,5-диарил-1,3-оксазола.

Результаты: В случае эритроцитов крыс, токсифицированных ПЕГ-400, наблюдается заметное снижение интенсивности флуоресценции всех использованных нами зондов. Обсуждаемое снижение интенсивности флуоресценции зондов свидетельствует об уменьшении количества молекул каждого из зондов, связавшихся с мембранами эритроцитов за один час инкубации, что свидетельствует об уменьшении скорости связывания зондов с мембранами. Такое уменьшение скорости связывания может быть объяснено формированием вокруг каждой липидной мембраны дополнительной защитной оболочки.

Вывод: Установленный факт формирования на поверхности мембран эритроцитов дополнительной оболочки из молекул полиэтиленгликоля методом флуоресцентных зондов можно трактовать как стабильный стандартизованый показатель свидетельствующий об отсутствии повреждающего действия на мемраны у объекта изучения, что требует подтверждения в дальнейших исследованиях.

Ключевые слова: химические факторы, полиэтиленгликоль, токсификация, эритроциты, биомембрана, липидный бислой, флуоресцентный зонд.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of coauthors of the article.

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