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NON-ANTIBACTERIAL EFFECTS OF ANTIBIOTICS: RESULTS OF OUR RESEARCH

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Abstract. Based on the idea of presence of non-antibacterial effects of different nature in antibiotics of various classes, we have studied the biophysical mechanisms of their interaction with eukaryotic cells using the model "antibiotic-erythrocyte" system as an example. We have researched the antibiotics used to treat mycoplasma infections: azithromycin (Sumamed, Croatia) – 1.34×10^{-4} mol/L, 1.34×10^{-5} mol/L, clarithromycin – 1.33×10^{-4} mol/L, 1.33×10^{-5} mol/L (Klacid, Abbott S.p.A); roxithromycin – 7.2×10^{-5} mol/L, 7.2×10^{-6} mol/L, josamycin – 1.21×10^{-4} mol/L, 1.21×10^{-5} mol/L, doxycycline hyclate – 7.8×10^{-5} mol/L, 7.8×10^{-6} mol/L, clindamycin – 1.4×10^{-4} mol/L, 1.4×10^{-5} mol/L, ciprofloxacin – 1.21×10^{-4} mol/L, 1.21×10^{-5} mol/L, ofloxacin – 1.1×10^{-4} mol/L, 1.1×10^{-5} mol/L, sparfloxacin – 1.02×10^{-4} mol/L, 1.02×10^{-5} mol/L (Sigma-Aldrich). It has been demonstrated by the use of biophysical methods of investigation that the antibiotics studied reveal a certain affinity with the human erythrocytes influencing morphofunctional organization of their molecules and the state of intracellular hemoglobin. As we've established by the method of osmotic hemolysis, the assessment of hemolytic activity of erythrocytes under the effect of antibacterial agents in therapeutic and sub-therapeutic doses *in vitro* has allowed us to find that all of the antibiotics tested, except for doxycycline, are weak hemolytic agents. Examination of the surface relief of the antibiotics-modified erythrocytes by way of scanning electron microscopy has shown a dose-dependent decrease of biconcave discocytes and an increase of the ratio of irreversibly deformed erythrocytes as against control. The data we obtained evidence a change of the surface architectonics of erythrocytes and a decrease of the polymorphism of the erythrocyte population. The most noticeable changes in the surface architectonics of red blood cells and in the red cell distribution width (anisocytosis) has been induced by doxycycline in both concentrations used. By the methods of absorption spectroscopy and potentiometric titration we have shown that the antibiotics studied can chemically modify buffer and conformational properties of intracellular hemoglobin. The nature of the modifier's effect is defined by its chemical structure and concentration in the incubation environment. An overview of the results of our own research, as well as the materials of the existing publications, allow one to view antibiotics as modifying agents, conditioning the typical reaction of peripheral erythrones to pathogenetic action. The article outlines our suggested scheme of processes of modification of key components of the erythrocyte membrane under the effect of antimicrobial drugs. It is concluded based on the foregoing that the choice of antibiotics should be made while taking into account their anti- and non-antibacterial properties.

Keywords: antibiotics, cell's transformation, erythrocytes, scanning electron microscopy, absorption spectrophotometry

The development of antimicrobial treatment was an important landmark of modern medicine. The use of antibiotics helped to treat potentially lethal infections; their wide dissemination and not at all times rational use in the developed and developing nations of the world have brought on unwanted consequences [1-7]. Research has revealed that the scale of effect of an antibacterial drug directly depends on its concentration in the site of impact [8]; some antibiotics in subinhibitory concentrations are able to achieve anti-inflammatory effect without an antimicrobial one [9], providing pathogenetic and immunogenic correction of infection [2, 3]. Due to the fact that metabolic processes taking place in blood cells reflect the response of cells of the whole organism under stress and clinical pathology [10, 11], we used red blood cells and hemoglobin obtained from blood donors as a model system.

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MATERIALS AND METHODS

We have researched the biophysical mechanisms of interaction of antibiotics used to treat mycoplasma infections (azithromycin – 1.34×10^{-4} mol/L, 1.34×10^{-5} mol/L (Sumamed, Croatia), clarithromycin – 1.33×10^{-4} mol/L, 1.33×10^{-5} mol/L (Klacid, Abbott S.p.A); roxithromycin – 7.2×10^{-5} mol/L, 7.2×10^{-6} mol/L, josamycin – 1.21×10^{-4} mol/L, 1.21×10^{-5} mol/L, doxycycline hyclate – 7.8×10^{-5} mol/L, 7.8×10^{-6} mol/L, clindamycin – 1.4×10^{-4} mol/L, 1.4×10^{-5} mol/L, ciprofloxacin – 1.21×10^{-4} mol/L, 1.21×10^{-5} mol/L, ofloxacin – 1.1×10^{-4} mol/L, 1.1×10^{-5} mol/L, sparfloxacin – 1.02×10^{-4} mol/L, 1.02×10^{-5} mol/L (Sigma-Aldrich), with the components of erythrocyte membranes and free hemoglobin.

We have studied the surface architectonics of erythrocytes of donor's blood by the method of scanning electron microscopy (SEM). Erythrocytes were pre-incubated with antibiotics for 60 mins. Then control and experimental samples were fixed by 2.5% solution of glutaraldehyde at a temperature of 4^0C for 1 h and dehydrated (ethanol solutions of 30-90%, acetone). The prepared suspension was applied on an aluminum substrate and dried in a thermostat at 37 ^{0}C. To ensure the electrical conductivity of objects they were sprayed with a thin film of gold. The prepared samples were viewed on scanning electron microscope JSM – 6380 LU (Japan) at an accelerating voltage of 20-25 kV.

By the method of potentiometric titration (the ionomer I-130, acidification of the medium to pH=3 with 0.1 n HCl) and absorbtion spectrophotometry (spectrophotometer «Shimadzu UV-2401 PC», λ = 200–650 nm, quartz cuvettes with l=10 mm) we researched structurally functional state of hemoglobin that was extracted from donor's blood by the method of osmotic hemolysis of erythrocytes pre-incubated with antibiotics for 60 mins. The amount of hemoglobin and the ratio of its individual ligand forms (oxyhemoglobin HbO_2, deoxyhemoglobin Hb and methemoglobin – MtHb) in the native and modified samples were determined by calculation formulas based on the values of optical density of hemoprotein solutions at wavelengths of 500, 569, 577 nm [12].

Statistical analysis. The data obtained were analyzed with "Statistica 6.0" software and standard packages of Microsoft Excel. The significance of differences between compared parameters was determined by Student's t-test ($p \leq 0.05$).

DISCUSSION

As we've established, the assessment of hemolytic activity of erythrocytes under the effect of antibacterial agents in therapeutic and sub-therapeutic doses *in vitro* has allowed us to find that all of the antibiotics studied, except for doxycycline, are weak hemolytic agents [13]. We have detected a dose-dependent effect of interaction of antibiotics with erythrocyte membranes, defined by the duration of interaction with the modifier. We have established that the antibiotics studied can chemically modify protein components of erythrocyte membranes and cause a decrease of biconcave discocytes (by 3-5% in the average, $p\leq0,05$) and an increase of the ratio of irreversibly deformed erythrocytes as against control (fig. 1).

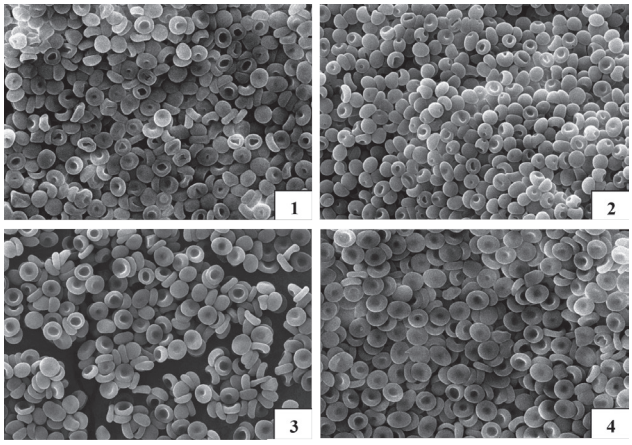

Fig. 1. Surface architectonics of erythrocytes modified by azithromycin, (1) 1.34×10^{-4} mol/L, doxycycline (2) 7.8×10^{-6} mol/L, clindamycin (3) 1.4×10^{-4} mol/L, roxithromycin (4) 7.2×10^{-5} mol/L (magnification x1,300)

It is well known that the morphologic sign of an erythrocyte's aging is a multi-step transformation of discocytes into echinocytes and more rarely – into stomatocytes [10], caused by the changes in the erythrocytes' cytoskeleton and cytomembrane [14]. "Intracellular oxidation processes are related to the changes of configuration of the inner part of the central pallor of the cells with the formation of conditionally polymorph stomata (CPS)" [15]. It's known that against the background of morphologic changes of erythrocytes, the de-energisation of erythrocytes, hypoxic damage, damage to the integrity and permeability of membranes, and membrane budding can also be registered [10, 14].

As it follows from Fig. 1, in case of 60-minutes incubation of erythrocytes with doxycycline [1] and azithromycin, discocytes with regular round pallor zone are replaced by cells with elongated CPS and star-shaped CPS. Apparently, these antibiotics induce accelerated "aging" of the erythrocytes, leading to functional cell failure.

Where a human organism is affected by various factors and a pathology develops, the change of configuration of erythrocytes, accompanied by a variation

of their sizes, shifts in the ratio of intracellular hemoglobin and an increase of dispersion of its intracellular distribution, becomes relevant for the diagnosis [15]. Emergence under the effect of doxycycline and other antibiotics of a larger, as compared to control (donor blood), amount of irreversibly deformed forms of cells, and a decrease in the amount of discocytes point at the disruption of the system's stability at the level of the entire cell, which contributes the modification of its functional state, and, accordingly, the erythrocytes' capacity for elastic deformation in the micro-circular bloodstream. Such changes of the structural and functional integrity of red blood cells have been noted in a number of publications [10, 11, 15], that also include data on the irregularity of protein or lipid composition of the erythrocyte membrane, disruption of the cation-transport systems of the membrane and other, which the authors of such publications define as the typical response of erythrocytes to pathogenic effect. It can be concluded then, that the changes of the biophysical parameters of erythrocyte membranes associated with age mirror the natural aging of the organism at the cellular and subcellular levels [10].

By the method of absorption spectrophotometry and potentiometric titration we have established that erythrocyte membranes are permeable for the antibiotics studied, which is manifested by the changes of the conformational properties of the molecules of intraerythrocytic hemoglobin. We have shown that the nature of the modifier's effect is defined by its chemical structure and concentration in the incubation environment. We have identified the absence of any interconnection between the quantification of affinity of the antibiotics studied with plasma proteins and the scope of changes of the conformational properties of intraerythrocytic hemoglobin [16].

It is known that the formation of the mechanism of the pharmacological effect of a drug largely depends on the polarity, form and size of the molecule [17-20]. As the substance's molecular weight increases, its activity and toxicity drop. If one assesses the activity of modifiers, taking into account the steric consistency of the molecules of the studied compounds, to the hydrophobic parts of the protein, it will be seen that macrolide antibiotics possessing greater molecular weight (800 Da on the average) and diversity of reactive groups as compared to fluoroquinolones (380 Da on the average), clindamycin (424 Da) and doxycycline (512 Da), surpass such modifiers by the degree of effect on the scope of buffer capacity of oxyhemoglobin solutions in all pH areas. Out of all antibiotics we've studied, roxithromycin that has the maximum degree of binding to plasma proteins (96%), demonstrated the greatest affinity to the oxyhemoglobin molecule, leading to a change of its structural rigidity during combination with ionogenic protein groups. However, the maximum reduction in the amount of methemoglobin in the hemoprotein solution (up to 0.05%) was found for Clindamycin, which induced the redistribution of the electronic density of the porphyrin ring, influencing the strength of the bond "heme-ligand".

Based on the results of our own research of the effect of antibiotics of various classes on the structural and functional condition of erythrocyte cells, as well as their contribution to the disruption of the organisation of membrane components and intracellular hemoglobin, and based on the literature in the field, we have produced a general conception describing processes of formation of structural changes in cellular structures under the effect of antibiotics (fig. 2).

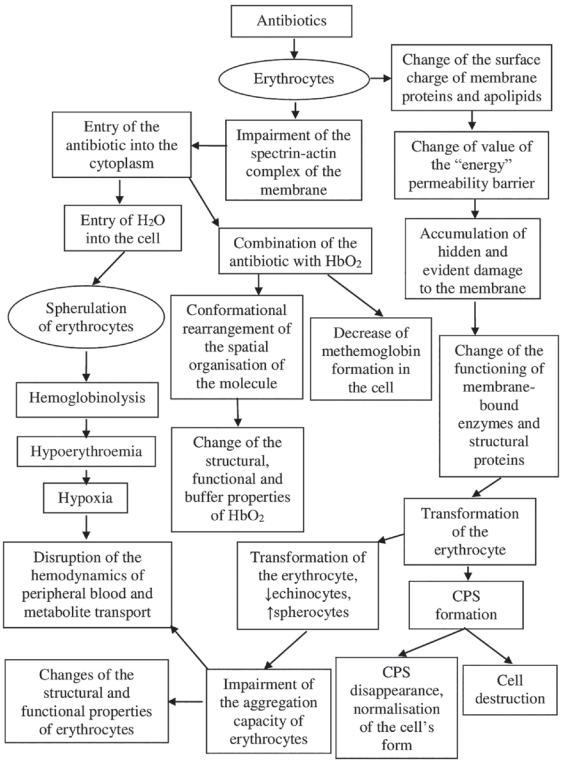

Fig. 2. General chart describing the processes of change in the key components of erythrocyte membranes under the effects of antimicrobial drugs

This chart is based on the representation of the physical and chemical aspects of interaction of human erythrocytes with antibiotics belonging to various classes, and reflects, on a step-by-step basis, the processes going on in erythrocyte cells under the effect of antibiotics from their entry into the cell up to the manifestation of the physiological effect. The capability of antibiotics that differ by their chemical struc-

ture, composition and molecular weight, of producing a similar effect on the key structural and functional characteristics of cellular components of erythrocytes has allowed us to present the main directions of this process. In interacting with the structural components of erythrocytes, antibiotics contribute to the accumulation of hidden and evident damage, leading to changes of the functioning of membrane-bound enzymes and structural proteins. This effect of antibiotics results in the transformation of erythrocyte cells, alteration of their surface architectonics and disruption of aggregation properties. The *in vitro* antibiotics-induced spherulation of erythrocytes can apparently result in their increased intravascular haemolysis *in vivo* with the disruption of hemodynamic of peripheral blood and transportation of metabolites. It follows from the chart that irrespective of the scenario, the entry of antibiotics into the erythrocytes eventually leads to the decrease of the red blood cells' capability of performing their key oxygen carrying function.

CONCLUSION

Thus, the heterogeneous changes of the structural and functional properties of erythrocytes under the effects of antibiotics identified by us, are the manifestation of their non-antibacterial action. The results of our own research as well as in the relevant publications, confirm the concept of the typical reaction of the peripheral link of the erythrone.

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НЕАНТИБАКТЕРИАЛЬНЫЕ ЭФФЕКТЫ АНТИБИОТИКОВ: РЕЗУЛЬТАТЫ СОБСТВЕННЫХ ИССЛЕДОВАНИЙ

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Аннотация. Основываясь на представлении о наличии у многих антибиотиков неантибактериальных эффектов различного рода, проведено изучение биофизических механизмов их взаимодействия с эукариотическими клетками на примере модельной системы «антибиотик-эритроцит». В работе использовались антибиотики, применяемые для лечения микоплазменной инфекции: азитромицин (Сумамед, Хорватия) – $1.34x10^{-4}$ моль/л, $1.34x10^{-5}$ моль/л, рокситромицин (Roxithromycin 90%, Sigma-Aldrich) – $7.2x10^{-5}$ моль/л, $7.2x10^{-6}$ моль/л, кларитромицин – $1.33x10^{-4}$ моль/л, $1.33x10^{-5}$ моль/л (Клацид, Abbott S.p.A); джозамицин – $1.21x10^{-4}$ моль/л, $1.21x10^{-5}$ моль/л (Josamycin, Sigma-Aldrich); доксициклин – $7.8x10^{-5}$ моль/л, $7.8x10^{-6}$ моль/л (Doxycycline hyclate 98% (TLC), Sigma-Aldrich); клиндамицин – $1.4x10^{-4}$ моль/л, $1.4x10^{-5}$ моль/л (Clindamycin, Sigma-Aldrich); ципрофлоксацин – $1.21x10^{-4}$ моль/л, $1.21x10^{-5}$ моль/л (Ciprofloxacin, ≥98.0% (HPLC) Sigma-Aldrich), офлоксацин – $1.1x10^{-4}$ моль/л, $1.1x10^{-5}$ моль/л (Ofloxacin, Sigma-Aldrich), спарфлоксацин – $1.02x10^{-4}$ моль/л, $1.02x10^{-5}$ моль/л (Sparfloxacin 98% (HPLC) Sigma-Aldrich). Используя различные биофизические методы исследования, показано, что данные антибиотики имеют определенное сродство к эритроцитарным клеткам человека, оказывая влияние на морфофункциональную организацию их мембран и состояние внутриклеточного гемоглобина. Методом регистрации осмотических эритрограмм выявлено, что тестируемые антибиотики, за исключением доксициклина, являются слабыми гемолитическими агентами. Посредством сканирующей электронной микроскопии установлено, что степень изменения цитоархитектоники, как и соотношения обратимо и необратимо трансформированных форм в субпопуляциях эритроцитов, определяются концентрацией препарата и временем его взаимодействия с клеткой. Наиболее выраженные изменения поверхностной архитектоники красных клеток крови, ширины их распределения по величине (анизоцитоз) индуцировал доксициклин в обеих использованных концентрациях. Методами протолитометрического титрования и абсорбционной спектроскопии показано, что тестируемые антибиотики модифицируют буферные и конформационные свойства внутриэритроцитарного гемоглобина. Представленный обзор результатов собственных исследований, а также данные литературы позволяют рассматривать антибиотики в качестве модифицирующих агентов, обуславливающих типовую реакцию эритрона периферического звена на патогенетическое воздействие. Описана разработанная нами схема процессов изменения главных компонентов в мембранах эритроцитов под воздействием антимикробных препаратов. На основании вышесказанного делается заключение, что выбор антибиотиков следует проводить с учетом имеющихся у них анти- и неантибактериальных свойств.

Ключевые слова: антибиотики, клеточная трансформация, эритроциты, сканирующая электронная микроскопия, абсорбционная спектрофотометрия

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