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GENDER-RELATED DIFFERENCES OF PHYSICAL PARAMETERS OF RAT ERYTHROCYTE MEMBRANES

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The gender-related differences of some biophysical parameters of rat erythrocyte ghosts are investigated by spectrophotometric method using the fluorescent probes ANS and pyrene. Data obtained show that the ghosts of male rat erythrocytes are more negative charged than females' and the straight of the bounding of peripheral proteins with lipid bilayer for female erythrocytes is less then for males'. The viscosity of female erythrocyte ghosts is more, but the compactness of the packing of phospholipids in lipid bilayer is less then in males', while the microviscosity of male erythrocyte ghosts is more then females'. These results testified that, in all probability, there are structural and functional gender-related differences in membrane level and these sex-related structural specificities of cell membranes can underlie the gender-related differences in pharmacokinetics and pharmacodinamics.

Gender-related differences – erythrocyte ghost – membrane structure

Սպեկտրաֆլյուրոմետրային մեթոդով հետազոտվել են արական և իգական սեռի առնետների էրիթրոցիտային ստվերների որոշ կենսաֆիզիկական չափանիշները՝ ֆլյուրեսցենտային զոնդերի՝ ԱՆՍ-ի և պիրենի, կիրառմամբ: Ստացված տվյալների համաձայն, իգական սեռի առանձնյակների էրիթրոցիտային թաղանթների բացասական լիցքն ավելի մեծ է, քան արականների մոտ, սակայն ծայրամասայի սպիտակուցների՝ լիպիդային երկշերտի հետ կապի ուժն ավելի մեծ է արական սեռի առնետների էրիթրոցիտների թաղանթներում: Թաղանթների մածուցիկությունը ավելի մեծ է իգական սեռի առանձնյակների մոտ, այն դեպքում, երբ լիպիդների փաթեթավորման կոմպակտությունն ավելի փոքր է, քան արական սեռի առնետների էրիթրոցիտային թաղանթներում: Ընդ որում, միկրոմածուցիկությունը արական առնետների էրիթրոցիտային թաղանթներում ավելի մեծ է: Ստացված տվյալները վկայում են այն մասին, որ առկա են էական կառուցվածքային և ֆունկցիոնալ տարբերություններ արական և իգական սեռի առնետների էրիթրոցիտային թաղանթների միջև, որոնք անհրաժեշտ է հաշվի առնել ֆարմակակինետիկական և ֆարմակադինամիկական հետազոտությունների ժամանակ:

Սեռ-կախյալ տարբերություններ – էրիթրոցիտային թաղանթներ – մեմբրանի կառուցվածք

Исследованы определенные биофизические параметры эритроцитарных теней у крыс мужского и женского пола спектрофлюорометрическим методом с помощью флюоресцентных зондов АНС-а и пирена. Показано, что отрицательный заряд теней эритроцитов у особей мужского пола менее выражен, чем у особей женского пола. Сила связи периферических белков с липидным бислоем у теней эритроцитов самок слабее, чем у самцов. Мембрана эритроцитов самок более вязкая, чем у самцов, но при этом упаковка липидов в бислое у них менее компактна. Микровязкость мембран у самцов выше, чем у самок. Получен-ные результаты дают основание полагать, что имеются существенные отличия в структурно-функциональном состоянии мембран самцов и самок, которые необходимо учитывать при фармакокинетических и фармакодинамических исследованиях.

Пол-зависимые различия – эритроцитарные тени – структура мембраны

Gender-related differences have been well described in pharmacokinetics and contribute to the interindividual variation in drug disposition, therapeutic response, and drug toxicity [2, 8, 9]. It is well known that drugs must traverse across biological membranes via simple diffusion or physiological transporters to produce therapeutic efficacy [13]. Gender-associated differences in transport processes for endogenous and exogenous substrates have been reported in various organs of the body, including kidney, liver, intestine, and brain, for rats, mice, and humans [1, 12, 20, 21, 22]. Additionally, kidney cortex brush-border vesicles isolated from female rats, exhibit increased membrane fluidity compared with ones isolated from male rats [4]. Gender-related differences in drug therapeutic response have not been extensively studied in membrane–level; however, differences in transport systems could contribute to interindividual variability in pharmacokinetics and pharmacodynamics.

It is well known that the functional state of cell membrane depends on the lipid-protein interactions in belier, which are determines the membrane structure [3, 5, 6]. We are suggested that the above mentioned sex-associated differences in drug therapeutic response are the result of the gender-related structural differences of membranes.

Erythrocyte membranes are convenient models for investigation of bilayer properties. Therefore, for testing of our hypothesis some biophysical parameters of rat erythrocyte membranes indicating the structural state of bilayer were investigated.

Materials and methods. The erythrocyte ghosts of 150-200 g male and female outbred white rats are served as an object of investigations. Blood is prepared by the method of cardio puncture [24]. The isolation of erythrocytes, preparation of their membranes and formation of ghosts was carried out by Dodge method in our modification [7]. Particularly, we have used the solution containing 0.145 M NaCl, 0.02 M Tris/HCl (pH 7.6) for red cell isolation, which allows us to increase the membrane outcome.

The biophysical parameters of erythrocyte ghosts are investigated by spectrophotometric method using the fluorescent probes 1-anilinonaftalene-8-sulfonate (ANS) and pyrene.

ANS is a water-soluble, non-penetrating probe with unit negative charge, which reacts in the sites of protein–lipid connections in the cell surface [10, 16, 23]. The bounding parameters of this probe with membrane are served as indicators for revealing the molecular reconstructions in membrane surface structure.

Pyrene is a hydrophobic, membrane-penetrating probe. The usage of this probe allows determining intramembrane changes, particularly the immersion degree of membrane proteins in lipids, polarity in bilayer, membrane viscosity and microviscosity [17, 18].

For getting the whole picture, fluorescence parameters of ANS and pyrene have measured in the same ghost samples. At the same time, for revealing the role of non-structured proteins, the isolation of erythrocytes and formation of ghosts have carried out in two samples: directly from the mass of erythrocytes after the tree-time washing in Tris-buffer (0.0145 M NaCl in 0.02 M Трис/HCl, pH 7.6) and from the mass of erythrocytes after the three-time washing in 0.9 %-NaCl solution.

ANS fluorescence of each ghost-containing simple has measured under the conditions of constant membrane-protein concentration (0.3 mg/ml) by titration with ANS (5-100 μM), and under the conditions of constant ANS concentration (5 μM) by titration with different protein concentrations (0.1-0.6 mg/ml). The obtained data have expressed in reversed coordinates, and the graphics have made by Klotz [23]. The rate constant of reaction (Kc) and the amount of ANS-bounding centers (N) have counted by formula of Scetchard [23]. The concentration of proteins in samples is determined by Lowry [14].

The measurement of fluorescent parameters of pyrene expressing the immersion degree of membrane proteins in lipid bilayer was carried out by method described in [19], in accordance to which the isolation of erythrocytes and formation of ghosts have carried out in two samples as described above.

The fluorescence of ghost-containing suspension in λ_{ext}= 284 nm and λ_{emis}= 334 nm is determined for estimation of fluorescence of tryptophanil groups.

The immersion degree of membrane proteins in lipid bilayer is estimated by inductive-resonance mechanism in triptophanil-pyrene system. Briefly, after the measurement of triptophanil fluorescence, 30 µl ethanol solution of pyrene with 100 µmol/l end concentration is added to the ghost-containing suspension.

The part of fluorescence of triptophanil groups arranging at a range not more than one Fester radius calculated by formula: $P = (F_0-F)/F_0$, where F_0 is a fluorescence of triptophanil groups before pyrene is added, F is the same parameter after the probe adding expressed in conventional units of fluorescence (CU).

The constant of the degree of relationship between the peripheral proteins with membranes calculated by formula: $K= |(P_1-P_2)/P_1|$, where P_1 is the value for ghosts obtained from erythrocytes washed in Tris-buffer, P_2 is the same parameter value for ghosts obtained from erythrocytes washed in NaCl solution.

Membrane microviscosity is estimated according to values of ratios I370/ I470, I390/ I470 fluorescence intensities of pyrene in λ_{ext}=284 nm. The increase of these parameters in erythrocyte membranes testifies the increase of microviscosity or the decrease of the hydrophobic volume of the zone of protein–lipid contacts [17, 18].

The values of ratios I370/ I470, fluorescence intensities of pyrene in λ_{ext}= 340 nm in all investigated samples are measured for estimation of viscosity of lipid bilayer.

The polarity in the environment of pyrene, which is the parameter estimated the compactness of the packing of phospholipids in lipid bilayer, is estimated by the I_{370}/ I_{390} ratio of pyrene in λ_{ext}= 340 nm.

All measurements have done in 1cm3 quartz cuvettes at the room temperature by the spectrometer Hitachi MPF-4 (Japan). The results are expressed in conventional units (CU) of fluorescence.

For each point of measurement the ghosts of erythrocytes isolated from 6 animals are used and each considering point is taken as an average of 10 measurements.

Statistical processing of results is done according to the Student's t-parameter.

Results and Discussion. As stated above, the isolation of erythrocytes and formation of ghosts is carried out in two samples: directly from the mass of erythrocytes after the three-time washing in Tris-buffer and from the mass of erythrocytes after the tree-time washing in 0.9%-NaCl solution.

The comparison of Kc for males and females in both cases of erythrocyte washing revealed the gender-related differences (tab. 1). So, as in the case of Tris-buffer washing, as after the NaCl washing, the values of investigated parameter for males is less in comparison with females by 34,48% and 58.54%, accordingly. The ANS molecule has a single negative charge [10, 23] and its affinity to membranes, consequently the mean of Kc, practically depends on the surface charge of ghosts. So, we can suggest that the surface of ghosts of erythrocytes of male rats are more negative charged than females'.

Table 1. The amount of ANS-bound centers (N) and the rate constant (K_c) of ANS binding reaction with the ghosts prepared from the mass of erythrocytes after the washing in Tris-buffer (0.0145 M NaCl in 0.02 M Tris/HCl, pH 7.6) and from the mass of erythrocytes after the washing in 0.9 % NaCl for female and male rats

ANS bounding parameters	Washing solution of erythrocytes	Female	Male
K_c ($\times 10^4$ mol^{-1})	Tris - buffer	0.58 ± 0.09	0.38 ± 0.04
	0.9 % NaCl	0.41 ± 0.05	0.17 ± 0.05
N ($\times 10^{-9}$ mol/mg proteins)	Tris - buffer	11.40 ± 1.85	11.77±1.54
	0.9 % NaCl	11.51 ±1.13	17.67±4.01

$p<0.05$

In accordance with the data introduced in the same table (tab. 1), the mean of K_c of ANS has changed depending of the way of washing. So, after the washing in 0.9 %-NaCl solution it has decreased by 29.31% in female and by 55.67% in male rat erythrocyte ghosts.

Taking into account that the NaCl washing of membranes influenced on the lipid belayer-peripheral proteins interactions, it is logically to suggest that the decrease of the affinity of membranes to ANS is a result of the changes of the amount or the conformational state of peripheral proteins. But it is obvious that the revealed change of K_c is more significance for males' (for 2 folds). In our opinion it is stated about the gender-related differences in membrane surface state, particularly in the lipid belayer-peripheral proteins interactions.

The analysis of the values of N show (tab. 1), that this parameter for the ghosts of erythrocytes of females was not changed depending on washing method, while for the males in the case of NaCl-washing we revealed the increase of the amount of ANS bounding centers with the ghosts by 50.13% in compare with ghosts of Tris-buffer washing erythrocytes. It is necessary to state, that the values of the amount of ANS bounding centers with the ghosts is the same for females and males in the case of erythrocytes washing in Triss-buffer, while the NaCl washing brings to the increase of N for male erythrocyte ghosts by 53.52% in compare with females'. These results allow us suggesting that the NaCl washing of male erythrocytes leads to the charge redistribution on the surface of cell membrane, which, on the one hand, brings to the increase of the amount of positive charged centers, which are the targets for ANS bounding. On the other hand, in all probability, the denudation of negative charged groups also takes place, which is clarify the decrease of affinity between ANS and ghosts, consequently Kc. On the bases of this point of view, the absence of the changes of investigated parameter for female ghosts depending on the way of erythrocyte washing, testified that the lipid belayer-peripheral proteins interactions in these membranes are differ from males'. We are suggesting that the straight of the bounding of peripheral proteins with lipid belayer for females is less then for males and even the Tris-buffer washing brings to their removing, thus in female ghosts for N we have not any differences depending on the washing way. It is following that the same values of N regardless of gender in the case of Tris-buffer washing is due to the changes of female ghosts in a result of removing of peripheral proteins. The investigations with the help of pyrene have shown that the immersion degree of peripheral proteins in erythrocyte membranes of female rats is more by 127,59% in compression with males' (fig. 1). In accordance with [19] , it is testified that the strength of relation between peripheral proteins and membranes for female erythrocyte ghosts is less than for males', which confirms our above mentioned suggestions.

So, in the bases of the obtained data we propose that the membrane surface structure of rat erythrocytes is gender-related.

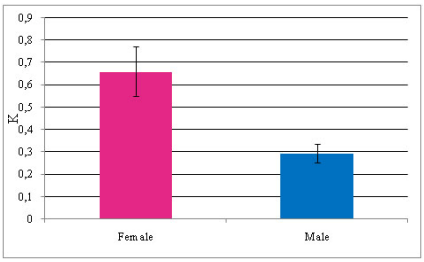

Fig. 1. Immersion degree (K) of peripheral proteins in erythrocyte membranes for the erythrocyte ghosts prepared from female and male rats (K_{Female}=0,66±0,11 K_{Male}=0,29±0,04).

The analyses of microviscosity, viscosity and polarity in the depth of membrane show that the values of investigated parameters do not depend on the way of the preparation of erythrocytes (tab. 2). I_{370}/ I_{470} and I_{390}/ I_{470} ratios of pyrene fluorescence in the excitation wavelength λ= 285 nm indicate the membrane microviscosity; I_{370}/ I_{470} and I_{370}/ I_{390}

in the excitation wavelength λ=340 nm indicate the viscosity and polarity in the depth of membrane, accordingly. Data introduced in the table above show, that these parameters are gender-related. So, the microviscosity of male erythrocyte ghosts is more then females by 127.08%, which can be interpreted if taking account above mentioned data testifying that the straight of relation of peripheral proteins with membranes is less for females' ghosts. The analyses of viscosity and polarity in the depth of membrane have revealed small, but reliable gender-related differences. So, they are more by 16.48% and 21.52% in female erythrocyte membranes, accordingly (tab. 2), which are testified that the viscosity of female erythrocyte ghosts is more, but the compactness of the packing of phospholipids in lipid bilayer is less then in males'.

Table 2. I_{370}/ I_{470} and I_{390}/ I_{470} ratios of pyrene fluorescence in the excitation wavelength λ=285 nm and I_{370}/ I_{470} and I_{370}/ I_{390} in the excitation wavelength λ=340 nm in the ghosts-containing solutions prepared from the mass of erythrocytes of female and male rats after the washing in Tris-buffer (0.0145 M NaCl in 0.02 M Tris/HCl, pH 7.6) and from the mass of erythrocytes after the washing in 0.9 % NaCl , *p<0.05

Fluorescence parameters	$\lambda_{emission}$	285нм		340нм	
	$\lambda_{extinction}$	I_{370}/I_{470}	I_{390}/I_{470}	I_{370}/I_{470}	I_{370}/I_{390}
Male	Tris-buffer	3,69±0,49	3,25±0,43	0,91±0,01	0,79±0,07
	0.9 % NaCl	3,41±0,48	3,46±0,56	0,91±0,01	0,79±0,08
Female	Tris-buffer	1,62±0,17	1,61±0,16	1,06±0,09	0,95±0,07
	0.9 % NaCl	1,49±0,32	1,38±0,19	1,07±0,11	0,96±0,06

Author in [15] introduced data about the lipid composition of erythrocyte membranes of male and female rats. According to them, there are significance gender-related differences in both phospholipids content and composition and in cholesterol content of membranes. In male erythrocyte membranes cholesterol content is more by 21.68% in compeer with females. It is well known that the membrane cholesterol content is defined the viscosity of membranes: as higher is it, as less is the membrane viscosity [5], which is observed in erythrocyte membranes of males' in compeer with females'. The polarity in the depth of membrane depends on the compactness of the packing of membrane phospholipids in inverse manner: as "distended" the packing, as polarity is higher. In accordance with our results, the compactness of the packing of phospholipids in lipid bilayer of male erythrocyte membranes is more then females'. On the other hand, the viscosity of female erythrocyte ghosts is more, but the compactness of the packing of phospholipids in lipid bilayer is less then in males', while the microviscosity of male erythrocyte ghosts is more then females'. These results testified that, in all probability, there are structural and functional gender-related differences in membrane level. So, we arrive at two conclusions. At first, the gender of animals is an important factor, which is necessary to take into consideration in membrane studies. And the second, the sex-related structure specificities of cell membranes can underlie the gender-related differences in pharmacokinetics and pharmacodinamics introduced above.

REFERENCES

1. *Anton F.M., Garcia Puig J., Ramos T., Gonzalez P., and Ordas J.* Sex differences in uric acid metabolism in adults: evidence for a lack of influence of estradiol-17 beta (E2) on the renal handling of urate. Metabolism, *35*, 343-348, 1986.
2. *Bonate P.L.* Gender-related differences in xenobiotic metabolism. J. Clin. Pharmacol. *31*, 684-690, 1991.

3. *Bledi Y., Inberg A., M.* Linial PROCEED: A proteomic method for analysing plasma membrane proteins in living mammalian cells. Briefings in Functional Genomics and proteomics, 3, 2, 254-265, 2003.
4. *Cerrutti J.A., Brandoni A., Quaglia N.B., and Torres A.M.* Sex difference in p-aminohippuric acid transport in rat kidney: role of membrane fluidity and expression of OAT1. Mol. Cell. Biochem., *233*, 175-179, 2002.
5. *Colin F.C., Gallois Y., Rapin D., Meskar A., Chabaud J.J., Vicariot M., Menez J.F.* Impaired Fetal Erythrocytes' Filterability: Relationship With Cell Size, Membrane Fluidity and Membrane Lipid Composition. Blood, 8, l79, 2148-2153, 1992.
6. *Cooper M.A.* Advances in membrane receptor screening and analysis. J. Mol. Recognit, 17, 286-315, 2004.
7. *Dodge J.T., Mitchell C., Hanahan D.* The preparation and hemical characteristics of hemoglobin-free ghosts of human erythrocytes, Arch. Biochem. and Biophis. 100/1, 119-130, 1963.
8. *Fletcher C.V., Acosta E.P., Strykowski J.M.* Gender differences in human pharmacokinetics and pharmacodynamics. J Adolesc Health, 15, 619-629, 1994.
9. *Harris R.Z., Benet L.Z., and Schwartz J.B.* Gender effects in pharmacokinetics and pharmacodynamics. Drugs, *50*, 222-239, 1995.
10. *Horie T., Sugiyama Y., Awazu S., Hanano M, 1-Anilino-8-naphthalene sulfonate binding site on human erythrocyte membrane using fluorescence lifetime and polarization, J. Pharmacobiodyn. 1982, 5/2, 73-80.*
11. *Janmey P.A., Kinnunen P.K.J.,* Biophysical properties of lipids and dynamic membranes, TRENDS in Cell Biology, doi:10.1016/j.tcb.2006.08.009, *2006.*
12. *Kleinman L.I., Loewenstein M.S. Goldstein L.* Sex difference in the transport of p-aminohippurate by the rat kidney. Endocrinology, *78*:403-40, 1966.
13. *Levy G.* Predicting effective drug concentrations for individual patients. Determinants of pharmacodynamic variability. Clin Pharmacokinet, *34*, 323-333, 1998.
14. *Lowry O., Rosebrough N., Farr A., Randal R.* Protein measurements with the Folin phenol reagent, J. Biol. Chem. 193/1, 265-275, 1951.
15. *Melkonyan M.M.* In Dissertation: Peroxide oxidation of lipids and application of antioxidants by acoustic stress, Yerevan, 1988.
16. *McLure J.A., Birkett D.J., Elliot D.J., Williams J.A., Rowl A., Miners J.O.*, Application of the Fluorescent Probe 1-Anilinonaphthalene-8-Sulfonate to the Measurement of the Nonspecific Binding of Drugs to Human Liver Microsomes, Drug Metabolism and Disposition. *39*, 9, 1711-1717, 2011.
17. *Nandy P., Choudhury M. D., Chakrabarti P*. Effect of sealing on the incorporation of pyrene in goat erythrocyte ghosts – A fluorescence study, J. Biosci. *5*, 2, 163-166, 1983.
18. *Novitskii V.V., Stepovaya E.A., Batukhtin A.V., Gol'dberg V.E., Kolosova M.V.* Erythrocyte membranes in patients with malignant tumors as shown by fluorescent probing, Bulletin of Experimental Biology and Medicine. *128*, 2, 857-859, 1999.
19. *Tereshchenko S. J., Prokhorenkov V., Prakhin E.* Patent of Russian Federation, № 2187112, 2002.
20. Orzes N., Bellentani S., Aldini R., Simoni P., Ferretti I., Lunazzi G.C., Sottocasa G.L., Tiribelli C. Sex differences in the hepatic uptake of sulphobromophthalein in the rat. Clin Sci (Lond). *69*, 587-593, 1985.
21. *Sibug R., Kuppers E., Beyer C., Maxson S.C., Pilgrim C. Reisert I.* Genotype-dependent sex differentiation of dopaminergic neurons in primary cultures of embryonic mouse brain. Brain Res. Dev. Brain Res., *93*, 136-142, 1996.
22. *Uhland-Smith A., DeLuca H.F.* 1,2,5-dihydroxycholecalciferol analogs cannot replace vitamin D in normocalcemic male rats. J. Nutr. *123*, 1777-1785, 1993.
23. *Vladimirov Yu.A., Dobrecov G.E,* In book: Fluorescence probes in the investigation of biological membranes, Moscow, Nauka, 1980.
24. *Weis H.J., Baas E.U.* Cardiopuncture in the rat for repeated sampling of blood and injection, Z. Gesamte. Exp. Med. *156* ,314-316, 1971.

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