Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

• Փորձարարական և տեսական հոդվածներ • Экспериментальные и теоретические статьи • • Experimental and theoretical articles •

Biolog. Journal of Armenia, 4 (72), 2020

SUPEROXIDE-PRODUCING ASSOCIATE BETWEEN NADPH OXIDASE AND NADPH CONTAINING LIPOPROTEIN (NCL) FROM HUMAN BLOOD ERYTHROCYTES AND LEUKOCYTES MEMBRANES: ACTIVATION OF IMMUNE CELLS BY NCL

R.M. SIMONYAN[1], S.G. CHAILYAN[1], V.A. CHAVUSHYAN[2], K.V. SIMONYAN[2], A.S. ISOYAN[2], M.A. BABAYAN[1*], G.M. SIMONYAN[1A], M.A. SIMONYAN[1]

**Corresponding author: Babayan M.A., Laboratory of Active oxygen species, H.Buniatyan Institute of Biochemistry NAS RA*
mbabayan@biochem.sci.am
[1] H.Buniatyan Institute of Biochemistry NAS RA,
[2]Orbeli Institute of Physiology NAS RA

From erythrocyte membranes (EM) and leukocyte membranes (LM) of human donor blood of group II the superoxide (O_2^-)- producing associate of NADPH oxidase (Nox) with NADPH-containing lipoprotein – NCL (Nox-NCL) was isolated and purified for the first time. Water solutions of the Nox-NCL have weak opalescence at pH 9.5. The specific content of the Nox-NCL from EM and LM is 3.6±0.04 mg/ml of erythrocytes, and from LM – 21.5±2.9 mg / ml of leucocytes ($p<0.05$, $n=6$). The specific O_2^--producing activity of the Nox-NCL EM is 30.2± 1,6 units/mg and 40,4±5,0 units/mg of these membranes, correspondingly ($p<0.05$, $n=6$). The mechanism of the production of O_2^- by these Nox-NCL associates contidioned by transfer of an electron from NADPH of this NCL to Fe(+3) of heme group of the Nox, then to molecular oxygen, reducing it to O_2^-.

Thus, NCL associated with isoforms of the Nox on the surface of EM and LM is a cofactor of this Nox for the production of superoxide radicals in heterogenous phase (on EM and LM) and homogenous phase (in solution), and separated NCL as activator of immune cells Nox was indicated for the first time.

Erythrocyte – leukocyte – membrane – NADPH oxidase – superoxide radicals – associate Nox-NCL – immune cells, activation

Առաջին անգամ մարդկային դոնորական արյան II խմբի էրիթրոցիտների թաղանթներից (ԷԹ) ու լեյկոցիտների թաղանթներից (ԼԹ) անջատվել և մաքրվել է սուպերօքսիդ (O_2^-)-գոյացնող ասոցիատ՝ կազմված ՆԱԴՐԻ օքսիդազից (Nox) և ՆԱԴՐԻ-պարունակող լիպոպրոտեինից-ՆՊԼ (Nox-ՆՊԼ): Nox-ՆՊԼ ասոցիատի ջրային լուծույթները pH 9,5-ում ունեն թույլ օպալէսցենցիա: Nox-ՆՊԼ-ի տեսակարար քանակը ԷԹ-երում և ԼԹ-երում համապատասխանաբար կազմում է 3,6±0,04 մգ/մլ և 21.5±2.9 մգ/1մլ, ($p<0.05$, $n=6$). ԷԹ-երից և ԼԹ-երից անջատած Nox-ՆՊԼ-ի տեսակարար O_2^--գոյացման ակտիվությունը համապատասխանաբար կազմում է 30.2±1,6 մ/մգ և 40,4±5,0 մ/մգ ($p<0.05$, $n=6$). Այդ ասոցիատներով O_2^- -գոյացման մեխանիզմը պայմանավորված է ՆՊԼ-ի ՆԱԴՐԻ-ից էլեկտրոնի փոխանցմամբ դեպի թաղանթային Nox-ի հեմային խմբի Fe(+3), որից էլ դեպի մոլեկուլային թթվածին՝ վերականգնելով այն մինչև O_2^- :

Այսպիսով, առաջին անգամ ցույց է տրվում, որ ԷԹ և ԼԹ մակերևույթին տեղակայված և Nox-ի հետ ասոսացված ՆՊԼ-ն՝ կոֆակտոր է Nox-ի համար հետերոգեն ֆազում (ԷԹ և ԼԹ մակերևույթին) և հոմոգեն ֆազում (լուծույթում)՝ սուպերօքսիդ գոյացնելու համար, իսկ առանձնացված ՆՊԼ-ն հանդիսանում է իմունային բջիջների Nox-ի ակտիվատոր է:

Էրիթրոցիտային – լեյկոցիտային թաղանթներ – ՆԱԴՐԻ օքսիդազ – ասոցիատ Nox-ՆՊԼ, իմունային բջիջներ – ակտիվացում

Впервые из эритроцитарных мембран (ЭМ) и лейкоцитарных мембран (ЛМ) донорской крови человека II группы выделен и очищен супероксид (O_2^-)-продуцирующий ассоциат NADPH оксидазы (Nox) с NADPH-содержащим липопротеином – НСЛ (Nox-НСЛ). Водные растворы ассоциата Nox-НСЛ имеют слабую опалесценцию при pH 9,5. Удельное содержание Nox-НСЛ из ЭМ и ЛМ составляют 3,6±0,04 мг/мл и 21.5±2.9мг/мл ($p<0.05$, $n=6$) соответственно. Удельная O_2^--продуцирующая активность Nox-НСЛ из ЭМ и ЛМ составляет 30.2±1,6 ед/мг и 40,4±5,0 ед/мг ($p<0.05$, $n=6$) соответственно. Механизм продуцирования O_2^- этими ассоциатами Nox-НСЛ обусловлен передачей электрона от NADPH из НСЛ к Fe(+3) гемовой группы Nox, далее к молекулярному кислороду, восстанавливая его до $O_2^{-\cdot}$.

Таким образом, впервые показано, что ассоциированный с Nox на поверхности ЭМ и ЛМ НСЛ является кофактором для Nox при продуцировании супероксидных радикалов в гетерогенной фазе (на ЭМ и ЛМ) и гомогенной фазе (в растворе), а отделенный НСЛ является активатором Nox иммунных клеток.

Эритроцитарные – лейкоцитарные мембраны – NADPH оксидаза – ассоциат Nox-НСЛ – иммунные клетки – активация

It is known that lipoproteins are important structural and functional components of the biomembranes and are involved in the synthesis of various bioactive compounds. Types of lipoproteins are present in the plasmatic membranes and membranes of intracellular formations of mammalian tissues and play an important role in the regulation of ion channels (including Kv7.2/Kv7.3). In this case, the lipoprotein cholesterol significantly modifies electrokinetic properties of human erythrocyte membranes with chronic cerebral ischemia and detection of phosphatidylserine in the erythrocyte membranes [1-3].

According to the currently available concepts, in the process of O_2^- production occurs combined electron transfer from cytosolic NADPH group of the cells with four Nox isoforms to the terminal and active Nox1 and Nox2 isoforms localized on the surface of the cell membranes for reduction of extracellular molecular oxygen to O_2^- [4].

Is there on biomembranes an alternative source of electrons (NADPH-containing component) for direct electron transfer to Nox isoforms for O_2^- generation ?

NADPH-containing superoxide-producing lipoprotein (suprol), which is activated by transition metal ions, was isolated from placental blood serum of women and mammalian blood serum for the first time [5-7]. Isolation and purification methods of NADPH containing lipoprotein associate with Nox of cell membranes of medicinal plants already available, in particular from the leaves of Stevia (*Stevia rebaudiana Bertoni*), and showed the stimulation phenomenon of O_2^--producing activity of Nox in heterogeneous and homogeneous phases [8]. Therefore, it will be possible for isolation and purification of the Nox-NCL associates from EM and LM.

The aim of the work to elaborate a method for isolation and purification of the Nox-NCL associates from EM and LM, as well as to separate of NCL from these associates and to determine the stimulation mechanism of NCL of the Nox and the mechanism of O_2^- production by these associates.

Materials and methods. **The isolation and purification of EM and LM from donor blood.**

The plasma of donor blood II group (20 ml each) was separated from red blood cells using 3% Dextrana-70 (“Loba Finchemie”, Germany), dissolved in saline [9]. After centrifugation and washing the precipitated red blood cells with saline and repeated centrifugation (2000 × g, 10 min), the precipitated red blood cells were hemolyzed with water (1:5 v/v). Further, by adding 0.05 M of HCI, the pH of hemolysate was adjusted to 5.6, by addition of 0,01 M HCI and the EM precipitated by centrifugation at 5500 × g, for 10 min. EM was washed with water (1:50 v/v) and

after centrifugation was homogenized with water by homogenizer. Leukocytes from blood plasma were also precipitated by centrifugation under similar conditions. After precipitation, the white blood cells with saline were homogenized with water, and then were frozen and thawed, after which LM were also precipitated at pH5.6 and collected by centrifugation. Traces of hemoglobin from EM and LM were removed by treatment with the mixture of ethanol and chloroform by volume ratios of 9:1. Separately, 1 ml of this mixture was added to 9 ml of membranes and incubated for 30 min under mixing conditions.

Hemoglobin turbidity was precipitated by centrifugation. Further, the EM and LM were centrifuged at 13.000 × g for 10 min, and the membrane precipitates were homogenized in water (1:10 v/v).

Isolation and purification of associates Nox-NCL from EM and LM

Associates Nox-NCL from EM and LM was isolated and purificated by universal method, using human ferrihemoglobin (Hb) for releasing of NCL and Nox from biomembranes [8]. In particular, for the breakdown of hydrophobic bonds of EM and LM, responsible for the retention of Nox- NCL on the surface of these membranes. Water mixtures of EM and LM were incubated at pH 9.5 and 37^0C for 1.5 hours in the presence of 50 mkM human Hb. After removal of "liberated" biomembranes by centrifugation at 13.000 × g, 10 min pH of supernatant was adjusted to 4.8 (by addition of 0,01 M HCI) and incubated at 4^0C for 30 min. Precipitate of the fraction of NCL from EM and LM was separately collected by centrifugation under presented conditions, washed with water (1:50 v/v) and after recentrifugation of the obtained precipitate of Nox-NCL associates from EM and LM was homogenized in water at pH 9.5 (40 ml for NCL from EM and 10 ml for NCL from LM). To remove possible traces of hemoglobin, these Nox-NCL associates were fractionated with ethanol and chloroform as reported above. As a result, opalescent solutions (at pH 9,5) of Nox-NCL from EM and LM were obtained. During ion-exchange chromatography of these solutions, on a column with DE-52 cellulose (also at pH 9.5) the Nox-NCL associates do not absorb on this column. Excess, non-NCL-associated total fractions of Nox1+Nox2 isoforms from EM and LM were eluted with 0.2 M potassium phosphate buffer (PPB) at pH7.4. Gel-filtration of Nox-NCL associates and Nox were carried out on separate columns with Sephadex G-100 at pH9.5. After desalination, Nox and Nox-NCL associates were subjected to vacuum lyophilization and stored in closed containers, under nitrogen atmosphere at $+4^0$C.

For separation of the NCL from Nox-NCL associates the Nox was inactivated by incubation with 0.05 M hydrogen peroxide at pH 4.8, at 37^0C for 20 min [10]. For removing of the traces of hydrogen peroxide, the NCL precipitates were washed twice with water (1:100 v/v), and adding 5×10(-7) M catalase and undergo ion-exchange chromatography on DE-52 cellulose, also at pH9.5. Traces of catalase, unlike NCL, are absorbed on this column.

Electrophoresis of the Nox-NCL associates was carried out in 10 % PAAG (Polyacrylamide Gel) for proteins of acidic or basic character.

The presence of a lipid component in the composition of these NCL was detected by determination the product of lipid peroxidation (malondialdehyde). Hydrophilic (methanol, ethanol, acetone) and hydrophobic (benzene, toluene, hexane) organic solvents were used to dissolve (separate) the lipid component from NCL.

Determination of superoxide-producing activity of Nox-NCL associates.

The O_2^--producing activity of these associates was determined by adrenaline method, which makes it possible to determine both prooxidant activity of associates and antioxidant activity of NCL [11]. For a unit of O_2^--producing activity we considered the amount of associates, which causes 50 % increase of the density of maximum absorption of adrenochrome (at 500 nm), formed during adrenaline oxidation by superoxide radicals. Specific O_2^--producing activity was expressed in the units/mg of associate. The reducing (antioxidant) activity of the NCL was determined by the inhibition of the oxidation of adrenaline.

The presence of NADPH in the NCL was determined by the spectrofluorimetric method. The NADPH emission peak in the composition of NCL was recorded at 430 nm, excited at 370 nm. NADPH solutions of a certain concentration were used as a control. Cellulose DE-52 (“Whatman”, England) and Sephadex G-100 (“Pharmacia”, Sweden) were used. During the investigation the spectrophotometer “Cary 60” UV/VIS, spectrofluorimeter “Perkin Elmer” (USA), centrifuge K-24 and K-70 (“Janetzki”, Germany) and homogenizer type MPW-302, (Poland) were used. Statistical analysis of the results by variational statistics method of Student-Fisher were carried out, determining the validation criterion (M±m, n=6).

Results and Discussion. Associates Nox-NCL from EM and LM does not break up during gel-filtration on the Sephadex G-100 and ion-exchange chromatography on DE-52 cellulose. As a result, excess of Nox isoforms from EM and LM, which are not part of the associates, are separated. The specific content of Nox-NCL associates from EM and LM is 3,6±0,04 mg/ml of red blood cells and 21,5±2,9 mg/ml ($p<0,05$, $n=6$) of white blood cells, respectively. Compared to the specific O_2^--producing activity of Nox-NCL associate from EM (represented as 30.2±1,6 units/mg of associate), the associate Nox-NCL from leukocytes is 40,4±5,0 units/mg ($p<0,05$, $n=6$). On the other hand, it is well known that the content of erythrocytes exceeds that of white blood cells about 1000 times. This suggests that the role of mature red blood cells is not limited only to the transfer of molecular oxygen to the cells, but perhaps also to the stimulation of the immune system [12-13]. As a result of the incubation of Nox-NCL associates with hydrogen peroxide the inactivation (denaturation) of Nox, and decrease of the optical absorption of α, β and γ bands, which are specific for Nox isoforms [10]. As a result of the cleaning by the above mentioned method, optical spectral indexes (A_{280}/A_{430}) of Nox-NCL associates, from EM and LM no longer decreased and ranged to 7.8 and 8.5, respectively. These Nox-NCL associates did not undergo to PAAG during electrophoresis and remained on the enter of the gel tubes in an aggregated state. Indirectly, the purity of these associates from EM and LM is evidenced by the fact, that during electrophoresis of the opalescent solutions of these associates on 10 % PAAG tubes strips of accompanying water-soluble proteins for acidic and basic nature were not detected. On the other hand, the symmetry of the elution diagrams of the Nox-NCL associates after gel-filtration through Sephadex G-100 also shows the purity of these associates.

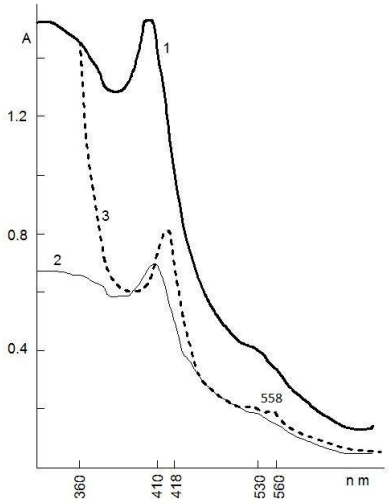

Fig. 1. Optical absorption spectra of opalescent solutions of Nox- NCL associates from EM (1) or LM (2) at pH 9.5. After reduction by sodium dithionite, the spectrum is obtained (3).

As indicated in fig.1, the optical absorption spectra of Nox-NCL associates from EM and LM are an overlay of the Nox and NCL spectra, with characteristic maximal absorption of Nox in an oxidized state (at 280, 360, 412, 530 and 560 nm), and after reduction by sodium dithionite crystals at 280, 360, 418, 540 and 558 nm. Weak absorption at 430 nm after removal of Nox isoforms is typical for associates (fig. 2).

At the same time, in oxidized and reduced states, the optical absorption spectra of Nox-NCL associates is preserved. This fact indicates that Nox as part of an associate retains its redox properties, acting as an electron transporter from NADPH of NCL on the

membrane surface to molecular oxygen, reducing it up to O_2^-. Interestingly, the color of the opalescent solution of Nox-NCL associates from EM and LM does not change under aerobic conditions. However, as a result of the reduction by electrons of NADPH of NCL of heme iron Nox in the NCL in the absence of air (vacuum or nitrogen atmosphere) the color of the opalescent solutions becomes crimson. This crimson color of associates quickly disappears after aeration of the solution. This indicates that, indeed, during the enzymatic production of O_2^- by associates, a rapid reduction and oxidation of the iron in the Nox heme group occurs. In fact, no irreversible chemical changes taking place of Nox-NCL associates from EM and LM. Unlike other O_2^--producing systems, the production of O_2^- by EM and LM associates in aerobic conditions *in vitro* occurs continuously and stops under anaerobic conditions. On the optical absorption spectrum of NCL from Nox-NCL associates of EM and LM, has a characteristic weak absorption at 430 nm in the visible region of the spectrum (fig. 2).

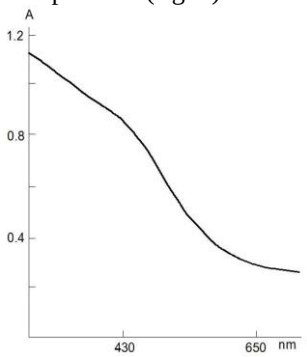

Fig. 2. Optical absorption spectrum of opalescent solution of NCL from associates of EM or LM at pH9,5 of donor blood after influence of hydrogen peroxide and catalase and ion-exchange chromatography on DE-52 ($p<0,05$, n=6).

NCL from associates of Nox-NSL of EM and LM due to NADPH in its composition, has only reducing (or antioxidant) properties: reduces potassium permanganate and suppresses the oxidation of adrenaline to adrenochrome. NADPH content in NCL from LM is slightly different from that of NCL of EM (>15-16 %).

Due to NADPH group associates Nox-NCL from EM and LM has a characteristic emission peak at 430 nm, with an excitation wavelength at 370 nm (fig. 3).

In the oxidized state, the optical absorption spectra of the total fraction of Nox1+Nox2 isolated from EM and LM has characteristic optical absorption maximumes: 560 nm (α), 530 nm (β) and 412 nm (γ-absorption band) and 360 nm (fig. 4).

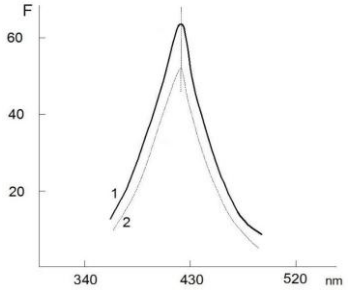

Fig. 3. Fluorescence spectrum of NADPH group as part of associates Nox-NCL from LM (1) and EM (2) of donor blood. F- is a fluorescence in relative units ($p<0.05$, n=6).

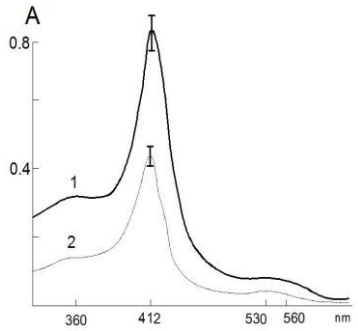

Fig. 4. Optical absorption spectra of total fraction of Nox1+Nox2 from EM (1) and LM (2) of donor blood (at pH 7.4), not associated with NCL, after reduction by sodium dithionite on the optical spectrum of these Nox, indicated the characteristic for the Nox absorption at 558 nm (α - band) ($p<0.05$, $n=6$).

Total fractions of Nox1+Nox2 isoforms from EM or LM are water-soluble proteins and have no background absorption, as shown in fig.1.

As shown in tab. 1, produced O_2^- by Nox-NCL associates from EM and LM oxidizes epinephrine to adrenochrome. On the contrary, NCL from EM and LM associates, as sources of electrons due to NADPH component, suppresses the oxidation of adrenaline and was indicated antioxidant properties.

Table 1. Relatively changes (%) of the decrease of the absorption (at 500 nm) of adrenochrome during oxidation of the adrenaline (2×10^{-4}M) under influence of 0,11 mg/ml NCL from associates of EM and LM or 2×10^{-8}M Cu,Zn-СОД and % of stimulation of the oxidation of adrenaline to adrenochrome by associate Nox-NCL (0,10 мг/мл) from EM and LM, in comparison with the 100% controls (indicies at the abcence of NCL or Nox-NCL)

Components	% of the suppression of adrenaline oxidation under the influence of NCL or SOD	% of the stimulation of the adrenaline oxidation (or formation of adrenochrome) under the influence of associate Nox-NCL
NCL from EM	92,7	-
NCL from LM	97,6	-
Associate Nox-NCL from EM	-	64,2
Associate Nox-NCL from LM	-	66,5

By means of Nox-NCL associate, as an O_2^- producing system on the surface of EM, the erythrocytes may also be components of the immune system. At the same time, on the cell surface, in particular, on EM and LM, isoforms of Nox are localized not separately, but with NCL, which plays both a functional role (produces superoxides) and a structural role (preserve the hydrophobicity of EM and LM).

The NCL from EM and LM (purified from traces of Nox), due to electron of NADPH group are activates the Nox isoforms of these membranes for the production of O_2^- both in a homogeneous phase (in solution) and in a heterogeneous phase (on the surface of these membranes). At the same time, as a bridge for the transfer of electrons from NADPH to O_2, not only the iron of the heme group of these Nox, but also the free Fe (III) ions, as in the case of suprol (superoxide-producing lipoprotein of mammalian blood serum) [5]. In fact, in the homogeneous and heterogeneous phase, NCL from Nox-NCL associates of EM and LM are the continuous source of electrons. Under aerobic conditions, Nox-NCL associates from EM and LM are natural, energetic and continuous

agents of O_2^- production, both in homogeneous and heterogeneous phases. This greatly elevated the prospects of using these natural associates to determine of the mechanisms of O_2^- action on various biosystems, including biomembranes, enzymes, RNA, DNA, cancer cells and microorganisms, as well as Covid-19.

NCL on the surface of EM and LM are the closest cofactors of Nox. The lipid component of these associates does not dissolve in hydrophobic or hydrophilic organic solvents. However, this lipid component undergoes lipid peroxidation to form malondialdehyde up to $1{,}5\times10^{-6}$ M and $1{,}2\times10^{-6}$ M ($p<0{,}05$, $n=6$) for EM and LM associates, respectively. Nox 3-6 isoforms are also localized on the membrane surface of intracellular micro- and nanoparticles (in particularly, ectosomes and exosomes), and may produce O_2^- by a direct mechanism.

By molecular weight, formation of opalescent solutions, lipid component (MDA) [14] and NADPH content, NCL from EM and LM associates are high density lipoproteins, as in the case of mammalian serum suprol [5, 6]. However, unlike suprol, NCL from associates of EM and LM, as components of these membranes, practically do not dissolve in hydrophilic and hydrophobic organic solvents and it is difficult to characterize them objectively yet. Perhaps insolubility of these NCL in organic solvents provides hydrophobicity of EM and LM [15].

Due to NADPH content, there are prospects for the use of NCL from EM and LM as potential activators of Nox on the cells membranes of immune system at immunodeficiency, when, as is well known, the superoxide-producing activity of leukocytes of various types decreases [16]. Quantitative and qualitative changes of the Nox-NCL associates from EM and LM can be used as new and sensitive diagnostic markers for various pathological conditions in the experiment and in clinical practice.

Nox-NCL associates from EM and LM separately and NCL from these associates EM and LM at pH 7.4 form an opalescent solution and practically do not lose activity in the saline medium. This elevates the possibility of infusing NCL into the blood stream in immunodeficiency in experiment.

Preparations of Nox-NCL associates from EM and LM, as well as NCL practically do not lose their basic activity at lyophilized state in closed vessels in a nitrogen atmosphere at 4^0C. This is an important factor for long-term use.

Thus, NCL associated with isoforms of the Nox on the surface of EM and LM is a natural cofactor of this Nox for production of superoxide radicals in heterogenous phase (on EM and LM) and homogenous phase (in solution) and the stimulation of the O_2^- -producing activity of the Nox immune cells by NCL were indicated on the first time.

REFERENCES

1. *Delgado-Ramírez, M., Sánchez-Armass, S., Meza, U., Rodríguez-Menchaca, A.A.* Regulation of Kv7.2/Kv7.3 channels by cholesterol: Relevance of an optimum plasma membrane cholesterol content, *Biochim Biophys Acta Biomembr*, 1860, 1242-1251, 2018.
2. *Fedin A.I., Vasilenko I.A., Badalyan K.R.* The effect of cholesterol on the electrokinetic properties of erythrocyte membranes, Zh Nevrol Psikhiatr Im Korsakova, 1*15*, 30-37, 2015.
3. *van Zwieten R., Bochem A.E., Hilarius P.M., van Bruggen R., Bergkamp F., Hovingh G.K., Verhoeven A.J.* The cholesterol content of the erythrocyte membrane is an important determinant of phosphatidylserine exposure, Biochim Biophys Acta, 1821, 1493-1500, 2012.

4. *Panday A., Sahoo M.K., Osorio D., Batra S.* (NADPH oxidases: an overview from structure to innate immunity-associated pathologies, *Cellular and Molecular Immunology, 12*, 5-23, 2015.
5. *Simonyan M.A., Karapetyan A.V., Babayan M.A., Simonyan R.M.* NADPH-containing Superoxide-Producing Lipoprotein Fraction of Blood Serum. Isolation, Purification, Brief Characterization, and Mechanism of Action, *Biochemistry* (Moscow), *61*, 671-675, 1996.
6. *Simonyan M.A., Karapetyan A.V., Galstyan D.A., Simonyan R.M., Babayan M.A.* Superoxide-producing lipoprotein as a factor of tumor growth suppression, enhancement of leukocyte number and acceleration of cell division in culture. *Biochemistry* (Moskow) *61*, 1578-1583, 1996.
7. *Alexanyan M.K., Simonyan G.M., Simonyan R.M., Arakelyan L.N., Alexanyan S.S., Simonyan M.A.* Antitumor and antistressor effects of suprol injected to white rats at sarcoma-45, *Medical Science of Armenia*, 52, 23-35, 2012.
8. *Isoyan A.S., Simonyan K.V., Simonyan R.M., Babayan M.A., Simonyan G.M., Chavushyan, V.A., Simonyan, M.A.* Superoxide-producing lipoprotein fraction from Stevia leaves: Definition of Specific Activity, *BMC Complementary and Alternative Medicine, 19*, 88-94. 2019.
9. *Neu B., Sowemimo-Coker S.O., Meiselman H.J.* Cell-Cell Affinity of Senescent Human Erythrocytes, *Biophys J., 85*, 75-84, 2003.
10. *Simonyan G.M., Grigoryan G.G., Simonyan R.M., Simonyan M.A.* High resistance of serum b558 cytochromes against hydrogen peroxide compared to other hemoproteins. Issues of military medicine, Collection of articles of the YSMU named after M. Heratsi, Yerevan, 48-51, 1999.
11. *Misra H.P., Fridovich I.* (The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem., *247*, 3170-3175, 1972.
12. *Morera D., Mackenzie S.* Is there a direct role for erythrocytes in the immune response? *Veterinary Research*, *42*, 89, 2011.
13. *Anderson H.L., Brodsky I.E., Mangalmurti N.S.* The Evolving Erythrocyte: Red Blood Cells as Modulators of Innate Immunity, *J. Immunol., 201*, 1343-1351, 2018.
14. *Vladimirov U.A., Archakov A.I.* Lipid peroxidation in biomembranes, "Nauka", Moscow (Russia), 1972.
15. *Simonyan R.M., Simonyan G.M., Simonyan M.A.* Method isolation of isoforms of NADPH oxidase (Nox) from biosystem License N2818 of Agency of the Invention Individual Property, Yerevan, RA, 2014.
16. *Simonyan, R.M., Aleksanyan, S.S., Engibaryan, A.A., Simonyan, M.A*. Suppression of hemoglobin-inducing releasing of NADPH oxidase from rats heart aorta cells membranes, *Issues in Theoretical and Clinical Medicine, 16*, 75-78, 2013.

Received on 25.09.2020