# THE EFFECT OF FREE AND BOUND NITRIC OXIDE ON BLOOD ENERGY METABOLISM

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**A.K. Martusevich,** PhD, Senior Research Worker, the Department of Experimental Medicine;
**A.G. Solovyova,** PhD, Research Worker, the Department of Experimental Medicine;
**S.P. Peretyagin,** D.Med.Sc., Professor, Head of the Department of Experimental Medicine

Nizhny Novgorod Research Institute of Traumatology and Orthopedics, Ministry of Health of the Russian Federation, Verkhne-Volzhskaya naberezhnaya St., 18, Nizhny Novgorod, Russian Federation, 603155

**The aim of the investigation** was to perform a comparative analysis of free and bound nitric oxide effects on erythrocyte energy metabolic parameters *in vitro*.

**Materials and Methods.** Two series of experiments were carried out. In the first series we processed blood samples (5 ml) by gaseous nitric oxide (800 µg/L) using three regimens of Plazon apparatus (Russia). In the second series we saturated three aliquot blood samples by 0.05; 0.1 and 0.2 ml of dinitrozyl iron complexes (DNIC) respectively (concentration of the compound was 3 mmol/L). We determined lactate dehydrogenase activity in direct and indirect reactions and lactate level in blood and erythrocytes. A number of derived coefficients were calculated to estimate energy metabolic changes.

**Results.** High concentrations of free nitric oxide were found to inhibit the activity of enzyme systems related to energy metabolism. On the contrary, DNIC have a stimulating effect on erythrocytes energy metabolism.

**Key words:** nitric oxide; dinitrozyl iron complexes; energy metabolism; lactate dehydrogenase.

As early as in 1989 B. Brune and E.G. Lapetina showed nitric oxide (NO) to influence the process of ribosilirizing some cytosolic protein with molecular mass 37 kDa [1], identified later as glyceraldehyde-3-phosphate dehydrogenase — a key enzyme of glycolysis [2, 3]. This effect was confirmed by J. Zhang and S.H. Snyder (1992) on the culture of neuronal cells [4], and further — in relation to other cellular pools and enzymes (phosphofructokinase of the pancreas islet cells and neurons, in particular) [5, 6]. A number of works is devoted to the participation of nitric oxide and NO-synthase in the adaptation to hypoxia and tissue hypoperfusion and even to neoplasmic process by modifying functioning of different links of tissue respiration [7–9]. However, the mechanism of this effect is not studied yet. Moreover, data about the character of the compound effect on the glucose intracellular transport and energy exchange are rather contradictory, the example being the skeletal muscle [9, 10]. These and other reports allow one to postulate the significance of nitric oxide effect on energy metabolism [10, 11], though the details are still remained obscure. The fact, that experiments carried out to clarify realization of bioregulatory function of NO, are based only on its indirect effect on the production level, makes the situation more complicated [1, 5, 9–11]. Taking into account the fact that in the organism and model biosystems, used for the majority of the appropriate investigations (cellular cultures), both enzymatic (by activation NO-synthase) and nonenzymatic synthesis of nitric oxide [11, 10] are present, and its release may also occur from the natural depots (S-nitrosothiols, dinitrozyl iron complexes [12–14], such approach often gives somewhat conflicting results. At the same time, investigations of the direct effect of NO on the biosystems are scare.

Besides, the principal problem is to determine physiological level of nitric oxide in biological systems [17], and consequently, it is difficult to choose adequately the range of exposing doses of NO to estimate their effect on energy exchange and processes of cellular respiration [18, 19]. Thus, we managed to ascertain by our previous investigations, that direct treatment of blood with high doses of the compound (800 ppm) causes suppressive action, shifting lactate dehydrogenase functioning towards a reverse reaction and leading to lactate accumulation, however, to understand the mechanisms of these shifts more detailed studies are necessary.

**The aim of the investigation** was to make a comparative analysis of gaseous (free) and bound (pooled) nitric oxide effect on the parameters of erythrocyte energy metabolism *in vitro*.

**Materials and Methods.** The pattern of whole preserved blood reaction to the action of free and deposited nitrogen have been studied. Two series of experiments were carried out: in the first one the effect of gaseous nitric oxide on the values of blood energy exchange was estimated, in the second series nitric oxide was in the form of dinitrozyl iron complexes (DNIC).

To carry on the experiment blood was divided into 4 portions (intact, which was not exposed, and 3 experimental ones, which were processed) in each series. In the first series a direct barbotage of the experimental blood samples (5 ml) by gaseous NO was performed at 3 power modes of the device used — minimal (min), average

For contacts: Martusevich Andrey Kimovich, phone +7 909-144-91-82; e-mail: cryst-mart@yandex.ru

(norm), and maximal (max). Cold plasma saturated with NO (with concentration of the substance in the gas flow in the selected conditions — 800 µg/L) was generated by Plazon apparatus (Russia). Exposure after the effect was 3 min.

In the second series 0.05, 0.1, and 0.2 ml of the freshly made water solution of DNIC was added to the experimental blood samples, respectively (compound concentration, determined by spectrophotometry according to the known molecular extinctions at the wavelength of 310 and 360 nm — 3 mmol/L). DNIC synthesis was performed according to the method developed by A.F. Vanin et al. (2005) [21]. Exposure after compound introduction was also 3 min.

The activity of lactate dehydrogenase (LDG) in the direct and reverse reactions was determined in the hemolysate of the washed eruthrocytes according to Kochetov's method (1980) in our modification [19, 22]. Protein content was estimated by modified Lowry's method. The level of lactate in blood plasma and erythrocytes was measured using analyzer Super GL Ambulance (Germany).

In order to assess the direction of the blood energy metabolic shifts under the influence of the preset physical and chemical factors a number of specific coefficients was used: a coefficient of energy reaction balance (CERB) [22], coefficient of substrate provision (CSP). Calculation of the given values was made using the following formulae:

$$CERB=\frac{LDGdir^2}{LDGrev^2}\cdot 100 \ [11],$$

where *LDGdir* — LDG activity in direct reaction, *LDGrev* — LDG activity in reverse reaction;

$$CSP=\frac{C\left(lactate\right)\cdot LDGdir}{LDGrev},$$

where *C* (*lactate*) — concentration of lactate in the blood plasma.

The results were processed using the program Statistica 6.0.

**Results and Discussion.** Taking into consideration the fact that the most studied molecular mechanism of NO action on the biological systems is the modification of catalytic properties of enzyme systems, we assessed LDG activity in direct and reverse reactions (Fig. 1). It was estimated that free and bound forms of NO produce a principally different action on the enzyme considered. Thus, in direct reaction of LDG nitric oxide, generated in all possible power modes of the Plazon apparatus, contributes to the moderate inhibition of enzyme activity (by 15–35% comparing to the control values; $p<0.05$), and no distinct relation between the power used and intensity of inhibiting effect was revealed (Fig. 1, *a*).

On the contrary, introduction of the pooled form of nitric oxide (DNIC) into the whole human blood results in considerable stimulation of the catalytic properties of LDG in direct reaction (by 38, 45.4 and 81.7% relative to the intact sample for 0.05, 0.1 and 0.2 ml of DNIC, respectively; $p<0.05$ for all cases). A marked dependence on the doses should be mentioned (the correlation level between the number of introduced DNIC and enzyme activity in the reverse reaction $r=+0.94$; $p<0.01$).

Investigations of LDG activity in reverse reaction allowed us to reveal the opposite dynamics: treatment of the biological fluid with NO-containing gas flow stimulates the catalytic properties of the enzyme (Fig. 1, *b*). And here again, like in the direct reaction no significant relations of the given parameter to the generator power was found. Thus, in all apparatus modes the increase of LDG activity in the reverse reaction was by 20.7–24.8% relative to the control sample ($p<0.05$). At the same time, introduction of the chemical analog of the natural pooled NO form — DNIC — into the blood samples did not cause any considerable shifts of LDG in the reverse reaction only in case of minimal compound dose used — 0.05 ml (0.15 µmol). If large quantities of DNIC were added into the biological liquid a prominent inhibiting effect was observed ($p<0.05$).

Such functioning dynamics of the enzyme considered makes it possible to suggest, that application of free NO in the concentration of 800 ppm causes an inhibiting effect on the given component of energy metabolism, making the process of aerobic glycolysis difficult, while introduction of the bound NO into the biofluid gives the opposite effect.

These findings are confirmed by the data of assessing intraerythrocytic level of lactate — one of the substrates of the enzyme studied (Fig. 2). It was estimated that the direct barbotage of the human whole blood samples by gaseous NO provides a significant increase of lactate in erythrocytes, the intensity of the assessed shift level value being practically unchanged with different modes of NO-generator. Taking into account the data on modifying the catalytic properties of LDG, we think, that the revealed accumulation of lactate within erythrocytes is the sign of forming energy deficiency under the influence of high NO concentrations, which confirms the data of different reports on inhibiting energy production processes in mitochondrions in indirect stimulation of NO synthesis [6, 20].

Our experiments determined that the effect of the pooled NO form does not influence significantly on the intraerythrocytic lactate concentration: its level grows essentially only when maximum quantities of DNIC are introduced into blood samples (0.2 ml=0.6 µmol). But at the same time, a direct relation between the value of this parameter and the quantity of the natural NO donor ($r=+0.93$; $p<0.01$) is noted.

To obtain an integral idea of the tendency of erythrocyte energy metabolism changes under the action of different NO forms calculation of some derivative coefficients was made. Thus, the dynamics of LDG functioning was assessed using the coefficient of energy reaction balance (Fig. 3). It was found, that treatment of the whole blood by gaseous NO results in moderate reduction of this parameter (2.1–3.5 times depending on the apparatus power applied; $p<0.01$). It additionally speaks of the formation of a marked energy deficiency in these samples with the shift of the catalytic LDG activity towards the reverse reaction. Blood saturation with the bound NO form, on the contrary, demonstrates dose-dependent increase of CERB, the rates of which are minimum, when 0.05 ml of

DNIC is introduced in the biological liquid, and when maximum of the selected agent doses is used (0.2 ml), the level of the value shows practically 5-fold increase ($p<0.01$). It indicates to the stimulation of the energy metabolism by nitric oxide, being observed by other authors in the condition of the physiological stimulation of NO-synthase activity [1, 5, 11].

Registration of the current lactate level during CSP analysis also allowed us to verify the effect of compared actions (Fig. 4). Thus, after blood barbotage by gaseous NO the value of the given assessment criterion does not practically differ from the controlled values due to the partial compensation of the marked prevalence of the LDG reverse reaction over the direct one, indicated by accumulation of lactate in erythrocytes. The power of NO generator directly influencing the gas flow rate, but not the concentration of NO, as the apparatus designers think, insignificantly alters CSP level.

As far as the bound form of NO is concerned, just like in case with CERB, dose-depending increase of the parameter value is registered, the greatest growth of this index being found beginning with 0.1 ml (0.3 µmol) of DNIC. It is important to note, that realizing with almost unchanged level of lactate in erythrocytes, this dynamics shows a predominant effect of the gradually releasing NO molecules on the LDG catalytic properties, which is confirmed by some indirect data in different reports [10].

Thus, the analysis of the energy metabolism parameters has also demonstrated different patterns of its reaction to the exogenous introduction of the free and bound NO forms *in vitro*, and what's more, in the first case we observed inhibition of this energy exchange component (energy deficiency formation), while in the second one — the increase of erythrocyte energy potential.

The variety of metabolic NO pathways, many of which are decoded at present (Fig. 5), tells about its dual molecular-cellular and organism effects. The results of the investigation performed reflect it to the full extent, underlining a key role of concentration and compound chemical formula in the effect development. Thus, high concentrations of free NO in addition to their direct damaging actions caused by peroxinitrite synthesis [8, 10, 12], inhibit the activity of many enzyme systems including those pertaining to energy metabolism. On the contrary, deposited forms of NO (DNIC) cause a marked stimulating effect on erythrocyte energy exchange because of the capability to release NO at an optimum rate due to the ability of binding with protein macromolecules and remaining in the blood for a long time even *in vivo* (in the biological fluid of the rabbit — for several days [14]). It should be noted that it is of dose-depending character, which is thought to be a positive pharmacological property of exogenous DNIC.

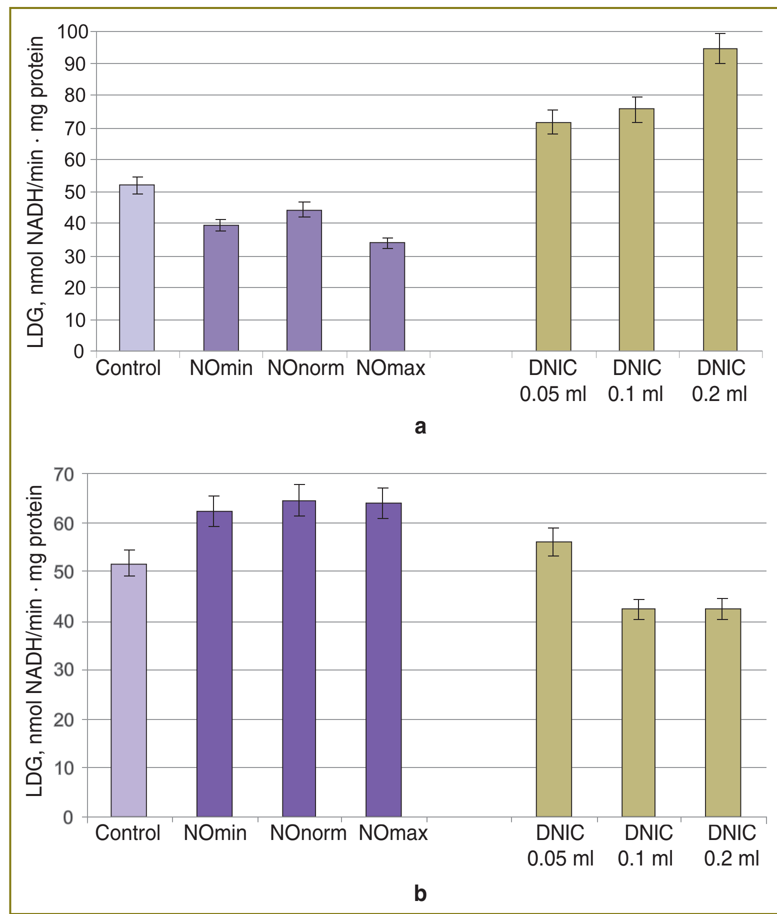


**Fig. 1.** Activity of erythrocyte lactate dehydrogenase in direct (*a*) and reverse (*b*) reaction under the action of different sources of nitric oxide *in vitro*

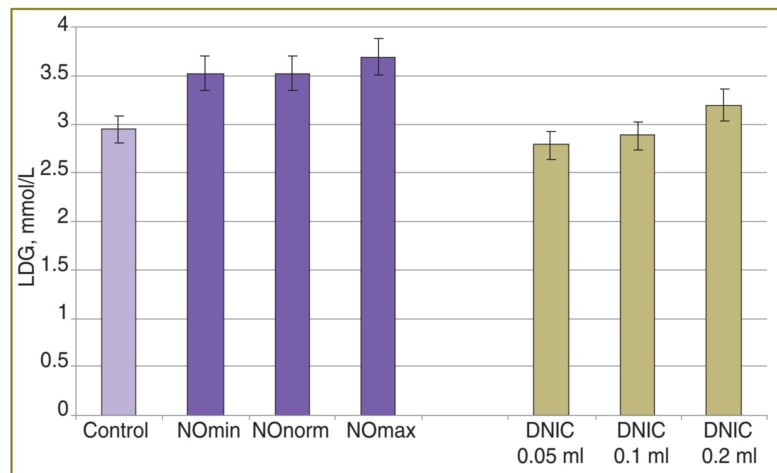


**Fig. 2.** Lactate level in erythrocytes in case of blood treatment by bound and free nitric oxide

**Conclusion**. The experiment findings show a marked direct action of nitric oxide on energy metabolism, the direction and significance of which are determined by the quantity and form of the acting agent.

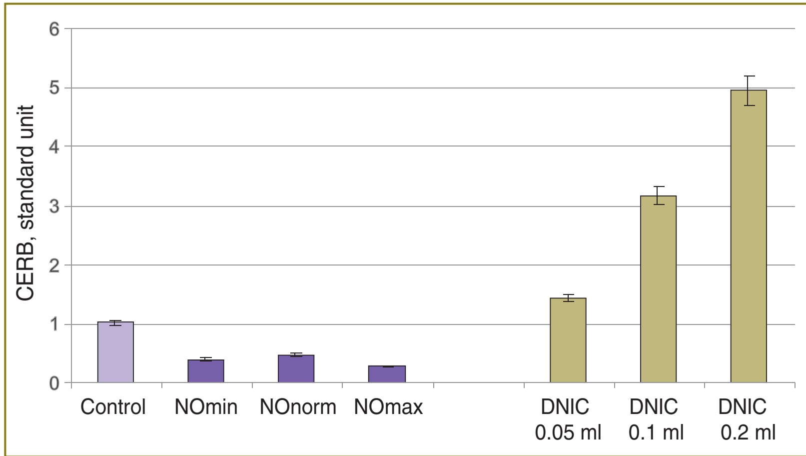


**Fig. 3.** The pattern of NO-associated shifts of the coefficient of energy blood reaction balance depending on the source of nitric oxide

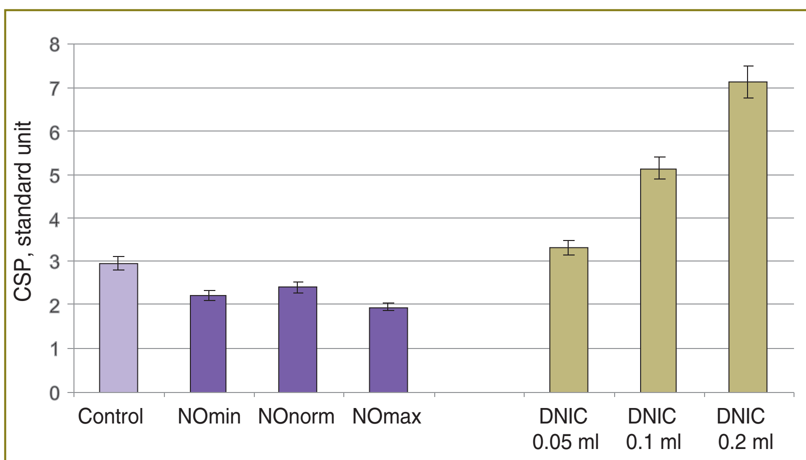


**Fig. 4.** The level of the coefficient of blood substrate provision treated by different NO

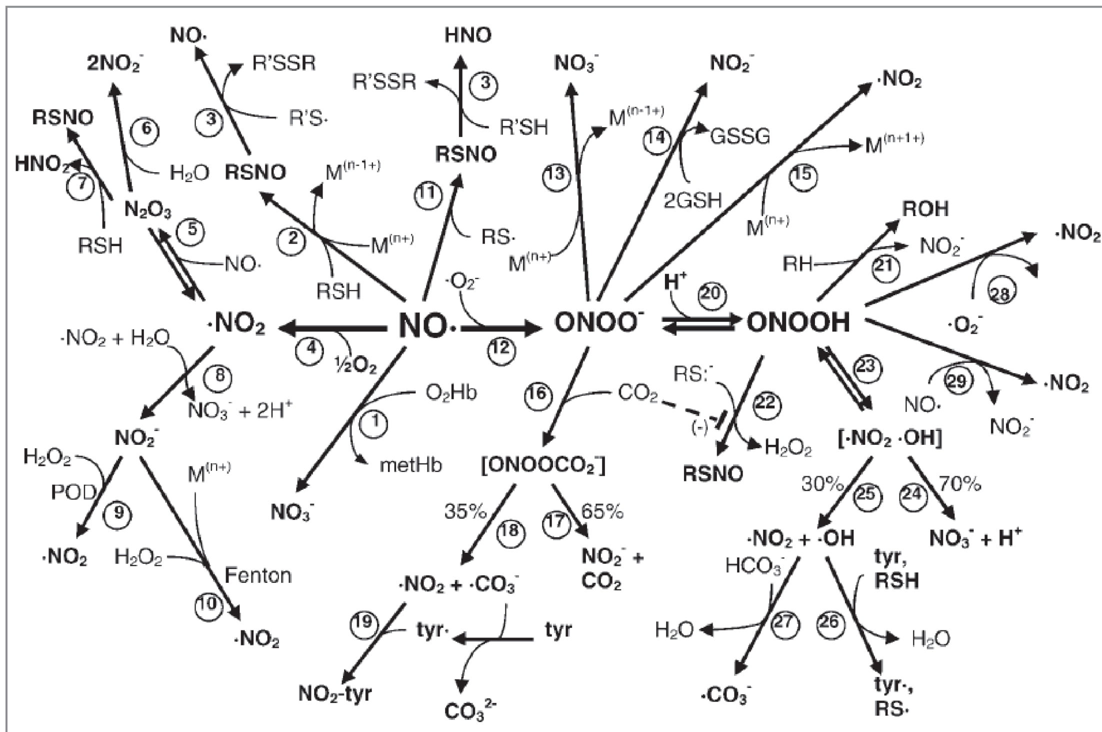


**Fig. 5**. Biochemestry of nitric oxide (according to Manukhina et al., 2006 [11])

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**Coflict of Interests.** The authors have no conflict of interests to disclose.

## References

**1.** Brune B., Lapetina E.G. Activation of cytosolic ADP-ribosyltransferase by nitric oxide-generating agents. *J Biol Chem* 1989; 264: 8455–8458.

**2.** Dimmler S., Brune B. Characterization of a nitric oxide-catalysed ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase. *Eur J Biochem* 1992; 210: 305–310.

**3.** Mohr S., Hallak H., de Boitte A., et al. Nitric oxide-induced S-glutathionylation and inactivation of glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* 1999; 274: 9427–9430.

**4.** Zhang S., Snyder S.H. Nitric oxide stimulates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase. *Proc Natl Acad Sci (USA)* 1992; 89: 9382–9385.

**5.** Almeida A., Moncada S., Bolanos J.P. Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. *Nat Cell Biol* 2004; 6: 45–51.

**6.** Tsuura Y., Shida H., Shinomura T., et al. Endogenous nitric oxide inhibits glucose-induced insulin secretion by suppression of phosphofructokinase activity in pancreatic islets. *Biochem Biophys Res Commun* 1998; 252: 34–38.

**7.** Mikhaylenko V.M. Izmeneniya energeticheskogo statusa opukholevykh kletok pri deystvii ekzogennykh oksidov azota [Energy status changes of tumor cells under exogenous nitric oxides]. *Sib Onkol Z — Siberian Journal of Oncology* 2009; Suppl 2: 137–138.

**8.** LeCras T.D., McMurthy I.F. Nitric oxide production in hypoxic lung. *Am J Physiol* 2001; 280(4): 1575–1582.

**9.** Young M.E., Radda G.K., Leigton B. Nitric oxide stimulates glucose transport and metabolism in rat skeletal muscle in vitro. *Biochem J* 1997; 322: 223–228.

**10.** Stanley W.C., Recchia F.A., Lopaschuk G.D. Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev* 2005; 85: 1093–1120.

**11.** Manukhina E.B., Downey H.F., Mallet R.T. Role of nitric oxide in cardiovascular adaptation to intermittent hypoxia. *Exp Biol Med* 2006; 231: 343–365.

**12.** Vanin A.F. Oksid azota v biomeditsinskikh issledovaniyakh [Nitric oxide in biomedical researches]. *Vestnik RAMN — Herald of RAMS* 2000; 4: 3–5.

**13.** Vanin A.F., Pisarenko O.I., Studneva I.M., et al. Deystvie dinitrozil'nogo kompleksa zheleza na metabolizm i kletochnye membrany ishemizirovannogo serdtsa krysy [The effect of dinitrozyl iron complex on metabolism and cell membranes of ischemic rat heart]. *Kardiologiia — Cardiology* 2009; 12: 43–49.

**14.** Vanin A.F. Dinitrosyl iron complexes with thiolate ligands: physico-chemistry, biochemistry and physiology. *Nitric Oxide Biol Chem* 2009; 21: 136–149.

**15.** Chazov E.I., Rodnenkov O.V., Zorin A.V., et al. Hypotensive effect of Oxacom containing a dinitrosyl iron complex with glutathione. Animal studies and clinical trials on healthy volunteers. *Nitric Oxide Biol Chem* 2012; 26: 148–156.

**16.** Giliano N.Ya., Konevega L.V., Noskin L.A., et al. Dinitrosyl iron complexes with thiol-containing ligands and apoptosis: studies with HeLa cell cultures. *Nitric Oxide Biol Chem* 2011; 24: 151–159.

**17.** Hall C.N., Garthwaite J. What is the real physiological NO concentration in vivo? *Nitric Oxide Biol Chem* 2009; 12: 92–103.

**18.** Martusevich A.K., Peretyagin S.P. Molekulyarnaya stereotipiya v realizatsii effekta nekotorykh lechebnykh fiziko-khimicheskikh faktorov: rol' NO [Molecular stereotypy in realization of the effect of some therapeutic physicochemical factors: NO role]. *Vestnik Nizhegorodskogo gosudarstvennogo universiteta im. N.I. Lobachevskogo — Vestnik of Nizhny Novgorod State University named after N.I. Lobachevsky* 2012; 2(Past 3): 205–210.

**19.** Martusevich A.K., Solovyova A.G., Peretyagin S.P., Mitrofanov V.N. Otsenka vliyaniya nekotorykh fizicheskikh faktorov na energeticheskiy metabolizm krovi in vitro [The assessment of the effect of some physical factors on blood energy metabolism in vitro]. *Biomeditsina —Biomedicine* 2013; 1: 103–108.

**20.** Tatsumi T., Matoba S., Kawahara A., et al. Cytokine-induced nitric oxide production inhibits mitochondrial energy production and impairs contractile function in rat cardiac myocytes. *J Am College Cardiol* 2000; 35(5): 1338–1346.

**21.** Vanin A.F., Lozinskiy V.I., Kapel'ko V.I. *Polimernaya kompozitsiya dlya sozdaniya stabilizirovannoy formy dinitrozil'nogo kompleksa zheleza i metod sinteza etoy formy* [Polymer compound for the development of a stable form of dinitrozyl iron complex and synthesis method of this form]. Patent RF №2291880. 2005.

**22.** Solovyova A.G., Zimin Yu.V. Novyy sposob otsenki dinamiki metabolizma krovi u bol'nykh s termicheskoy travmoy [A new estimation method of blood metabolism dynamics of patients with heat injuries]. *Sovrem Tehnol Med — Modern Technologies in Medicine* 2012; 2: 116–117.