OXIDATIVE – NITROSATIVE MODIFICATION OF HEMOGLOBIN IN ERYTHROCYTES OF RATS ON THE MODEL OF ALCOHOL INTOXICATION

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S u m a r y. The ratio of hemoglobin ligand forms has been studied on the model of rat alcohol intoxication. The findings show an increase in methemoglobin and a decrease in oxyhemoglobin in the peripheral blood of rats. Simultaneously, there is a decrease in hemoglobin affinity with oxygen under alcohol intoxication. It is caused by the modification of protein components of haemoglobin by acetaldehyde (alcohol metabolite) and the formation of the nitrozyl form of hemoglobin. It has been also revealed that L-arginine exerts a corrigent effect on the functional state of hemoglobin under alcohol intoxication.

K e y w o r d s**:** erythrocytes, alcoholic intoxication, hemoglobin.

INTRODUCTION

Peripheral blood serves as a significant indicator of the pathological state of an organism caused by factors of endogenous or exogenous origin. Alcohol and intermedates of its metabolism is one of such factors [6]. The study into compensatory-adaptative reactions with participation of the peripheral blood system on the action of toxic compounds (including ethanol and acetaldehyde, which is an intermedate of its metabolism) is of crucial importance in understanding the mechanisms for the development of pathologic conditions and adaptation to them at the cell and tissue levels.

The respiratory function of blood is carried out by hemoglobin (Hb) [2], which is the main protein of red blood cells, and consists of supplying cells of the whole body with molecular oxygen (O2) in quantities adequate to their metabolic needs [8]. Normal oxygenation of tissues is directly dependent on the intensity of O2 in capillaries, which should be high enough to release O2 from capillaries and diffuse into tissues. Under hypoxemia, when pO2 in capillaries is not high enough to provide cells with oxygen in accordance with their metabolism, hypoxia of tissues can occur.

Hemoglobin can form stable ligand forms with O2 and other compounds. Depending on the type of ligand (O2, CO, H2S, etc.), respectively, oxyhemoglobin (HbO2), carboxyhemoglobin (HbCO) and sulfhemoglobin (SHb) are formed. When methemoglobin (MetHb) is formed, the ligand is replaced by a water molecule. The interaction between nitric oxide (NO) and Hb in erythrocytes is of special interest since those cells have their own synthesis of NO.

NO is generated when in L-arginine is oxidized to L-citrulline by NO synthase enzyme (NO-synthase; NOS; L-arginine, NADPH:oxygen oxidoreductase [EC 1.14.13.39]). Nitroso compounds can be formed after the exogenous introduction of NO molecules donators (nitroprusside, nitroglycerin) or the NOS substrate (L-arginine) [5, 11].

The function of NO is multifaceted. It can change from physiological to cytotoxic under con-

ditions of both overproduction and deficiency in NO formation in pathologies of different genesis.

The overproduction of NO suppresses cyclooxygenase, enhances the cytotoxicity of hydrogen peroxide (Fenton reaction), and initiates cell apoptosis. Active NO intermedates are produced in the presence of oxygen, peroxide nitrite ($ONOO^-$) in particular, which is a powerful antioxidant and exerts a cytotoxic effect [5].

Additionally, NO bioactivity is limited by its fast oxidation to nitrites or nitrates. When interacting with hemcontaining proteins, NO is oxidized to nitrate (NO_3^-), which is restored to NO_2^- by xanthine oxidase, bacteria of the abdominal cavity and other tissue enzymes.

If there is deficiency in NOS substrate (L-Arg), super-oxide anion radical (O_2^-) may start producing the cascade of oxidative reactions in the cell instead of NO. Furthermore, the enzymatic synthesis of NO decreases if the partial pressure of oxygen in tissues falls below 30 mm Hg. In that case, another nitrite reductase way of NO formation (with the participation of hemoglobin) is used in the organism.

The pharmacological "imitation" of L-arginine/NO system activation through L-arginine (L-Arg) – the main NO-synthase substrate introduction or through its inhibition by L-NAME (methyl ester of Nω-nitro-L-arginine) may help find ways of efficient antistress protection and decipher molecular mechanisms for increasing adaption abilities for the organism under pathologies.

MATERIALS AND METHODS

Outbred white rats with the initial weight of 200-250g were used for experiments performed in accordance with the national "General Ethical Principles of Animals Experiments" approved by the First National Congress on Bioethics (Kyiv, Ukraine, 2001), which are consistent with the provisions of the European convention on the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, France, 1985). Moreover, all the experiments were carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive date 24 November 1986 for the Care and Use of Laboratory Animals (86/609/EEC), approved by the Local Ethics Committee. All the animals received a standard diet and had free access to water.

Experimental chronic alcoholic intoxication in rats was caused by the daily introduction of alcohol for 14 days *per os* in a dose of 6 g/kg [3, 4].

Equivalent glucose solution was administered to rats in a dose of 10.2 g/kg to save the energy value of the diet.

L-arginine (L-arg) ("Reanal", Hungary) in the concentration of 1.25 g/l and L-NAME (Nω-nitro-L -arginine methyl ester, "Beckenham", UK) in the concentration of 70 mg/l were introduced to animals *per os* in drinking water during 14 days simultaneously with the induction of alcoholic intoxication during *in vivo* experiments.

Six groups of animals were used for the experiments: 1 – Control (C); 2 – C + L-arg; 3 – C + L-NAME; 4 – Alcoholic intoxication (AI); 5 – AI + L-arg; 6 – AI + L-NAME.

The ligand forms of Hb have been determined by absorption spectroscopy method. The affinity of Hb with oxygen has been determined according to the technique in the special saturator [1, 9]. The reaction of nitrite anion reduction with the participation of deoxyhemoglobin (RHb) was initiated by the addition of 6 mM of NaNO2 to the solution of deoxyhemoglobin in the spectrophotometric cuvette. For analyses connected with hemoglobin nitrosation, the obtained MetHb solution was reduced to RHb adding sodium hydrosulfite in the spectrophotometric cuvette. Nitrosylated Hb was produced according to the technique [7].

The results were processed by the methods of variation statistics with the definition of probable changes by Student t-test. The difference was considered reliable at $P < 0.05$.

RESULTS AND DISCUSSION

The rate of NO conversion into NO3- anion is higher in whole blood than in plasma, which confirms the active participation of formed blood elements in NO metabolism [5].

NO2- - ions formed in plasma due to intravascular NO oxidation can be inversely diffused into the red blood cells, interact with Hb and under go oxidative and reduction reactions. Those reactions in erythrocytes are important modulators of intravascular NO bioactivity, especially under physiological hypoxia since the presence of various Hb compounds with NO differently affects Hb affinity with oxygen [10].

The determination of hemoglobin affinity with oxygen by the method of making dissociative curves of oxyhemoglobin (CDO) is considered to

be a universal approach to the oxygen-transport function of blood, oxygen-binding abilities of Hb and hence tissue oxygenation. Reduced oxygen affinity of Hb may be associated with increased sensitivity to 2,3-diphosphoglycerate (2,3-DFG), an essential intraerythrocytic regulator of hemoglobin affinity with oxygen [12]. Thus, we conducted the study into hemoglobin affinity with oxygen in hemolysates of rat erythrocytes pre-clearing the samples from 2,3- DFG (Table 1).

Parameter P50 analysis in Table 1 shows that under AI conditions there is a decrease by 13.3% Hb affinity with oxygen compared with the control (Table 1).

Table 1. L-arginine and L-NAME effects on the functional state of blood hemoglobin in rats in the control, under AI (%, n= 4 - 7)

Animal group	P_{50}, mm Hg	Ligand forms	
		HbO_2	MetHb
Control	30.00 ± 0.63	90.00±1.73	2.77±0.93
C + L-arginine	33.75 ± 1.64*	89.70±1.33	4.08±0.36
C + L-NAME	26.92 ± 1.16*	91.70±3.06	3.11±0.42
AI	34.00 ± 1.05*	85.20±2.27*	9.78±3.40*
AI + L-arginine	29.92 ± 0.80**	92.60±2.51**	2.13±0.24**
AI + L-NAME	20.42 ± 0.80**	88.70±0.75	5.90±0.17*

* – reliable difference compared with the control, P<0.05; **– reliable difference compared with AI, P<0.05.

In C+ L-NAME and AI + L-NAME animal goups, the effect was opposite and Hb affinity with oxygen increased compared with control and AI groups.

When L-arginine was administered to healthy rats, there was a reliable decrease in Hb affinity with oxygen by 12.5% compared with the control group of animals. Under AI, L-arginine consumption led to a decrease in parameter P_{50}, which testifies to an increase in Hb affinity with oxygen within control values.

Under the pathology Hb affinity with oxygen may change due to changes in the ratio of its ligand forms, production of nitrosothiols and additional hemoglobin oxidation products. The proportion of MetHb in the blood of animals under AI increased compared with the control 3.5 times. A decrease in the proportion of HbO2 caused in Hb oxygen capacity, which exacerbate the hypoxic state of organism under AI. Therefore, Hb affinity with oxygen under AI is so changing that even an increase in the proportion of MetHb in hemolysates does not cause its increase.

As for L-NAME solution, its consumption by the control group of rats was not accompanied by considerable changes in redistribution of hemoglobin ligand forms. Simultaniously, the consumption of L-NAME by animals under AI was accompanied by a twofold increase in the proportion of MetHb compared with the control (Table 1).

The findings have revealed that the consumption of L-arginine by the control rats does not change the ratio of hemoglobin ligand forms. However, the consumption of L-arginine under AI leads to an increase in the proportion of HbO2 and a decrease in MetHb quantities within the control values (Table 1). L-arginine consumption produced distinct corrigent effects on those indexes under AI.

A decrease in Hb affinity with oxygen during a high content of MetHb may be explained by the formation of acetaldehyde-modified Hb complexes or Hb compounds with NO.

To check the assumptions, we have researched into affinity of hemoglobin with oxygen in partically healthy donors, as well as the content of its ligand forms under various concentrations of acetaldehyde (Table 2). When AcA solution was added to hemolysates of red blood cells, there was increase in index P50, just as in vivo. It shows a significant role of EtOH - AcA metabolite in disturbance of the respiratory function of hemoglobin under AI.

Table 2. The degree of hemoglobin saturation with oxygen and the content of ots ligand form in the blood of healthy donors under various concentrations of AcA (M ± m)

Experimental options	n	P_{50}, mm Hg	Ligand forms	
			HbO_2	MetHb
Control	9	28.0 ± 0.92	94.2 ± 4.2	0.4 ± 0.2
C+ AcA 0.25 mM	5	32.0 ± 0.56*	94.0 ± 6.0	0.8 ± 0.5
C + AcA 0.50 mM	5	31.2 ± 0.86*	88.6 ± 3.6	4.1 ± 0.2*

* – reliable difference compared with the control, P<0.05

Under AcA, there is a decrease in HbO_2 content in all the experimental options. The decrease in the proportion of HbO_2 has been already observed at AcA concentration of 0.5 mM. The higher was AcA concentration, the higher was an increase in the proportion of MetHb. It should be noted that AcA addition to erythrocyte hemolysates *in vitro* at a concentration of 0.5 mM has the same results as *in vivo* under AI. When 0.5 mM of AcA was added, there was an increase in the proportion

of MetHb 10.3 times compared with the control (Table 2). Those experiments give grounds to argue that AcA directly interacts with hemoglobin.

In addition to that, Hb affinity with oxygen in blood can also influence the connections of hemoglobin with NO. It is known that, NO is linked with the heme part of Hb producing nitrosulhemoglobin (HbFe2+NO) or with cysteine at position 93 of ß-chain globine producing nitrosohemoglobin (SNOHb), which provides NO transport and release [5, 11]. The interaction between NO and Hb is important for regulating the functioning of those both molecules in vivo. It is known that, methemoglobin and SNOHb reduce index P50 of hemoglobin saturation with oxygen, whereas HbFe2+NO increases it.

Having analyzed the results of Hb affinity with oxygen under AI, when L-arginine was consumed, it may be assumed that exogenous introduction of NOS substrate induces a number of reactions leading to the formation of NO de novo and changes in its depositing with hemoglobin.

The study into the interaction of hemoglobin with NO is three orders of magnitude higher than that of O2, which suggests its competition with oxygen for the corresponding areas of the molecules of reduced or even partially oxidized hemoglobin. It is known that, NO binds to heme iron covalently, whereas only weak interaction is formed between O2 and Fe2+. While intercting with RHb, the main amount of NO is eliminated from the bloodstream and stored in the form of nitrosyl- and nitrosohemoglobin.

Significant changes in absorption spectra of RHb transition into HbFe2+NO have been detected in the visible area of 400 – 420 nm. The electronic spectrum of HbFe2+NO in options under AI is characterized by a hyperchromic effect within the Sore band, the maximum of which is at the wavelength of 417.7 nm (Fig. 1).

Having analyzed the area of the absorption spectrum that may confirm the nitrosylation of globin by cysteine at position 93 of β-chain of globin, it was noted that the intensity of the band in this area under AI was higher compared with the Hb band of the control animals (Fig.1).

When L-arginine was consumed by the rats under AI, there was decrease in absorption intensity of HbFe2+NO in the spectrum area (band Sore) compared with Hb in animals under AI without L-arginine consumption. When L-arginine was consumed by rats under AI, the approximation of characteristic values of Hb absorption spectrum in SNO-Hb zone to those in control was observed. Under those conditions, SNO-Hb can perform a role of a critical O2 supply factor since it acts as an «alosterically controlled nitrogen oxide buffer», which exchanges its NO-group with thiols of the medium, including glutathione, thus affecting the flow of blood as a vasodilator [11].

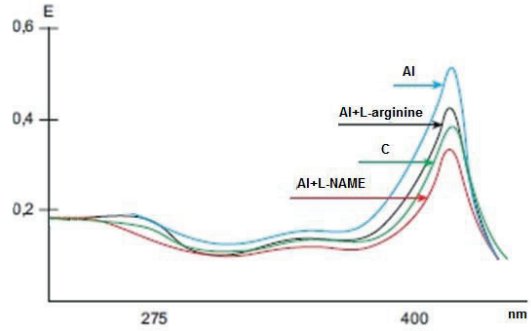

Fig. 1. Typical NOHb electronic spectra obtained exogenous nitration of hemoglobin samples *in vitro* using $NaNO_2$ in the control, under AI and through L – arginine or L – NAME introduction

CONCLUSIONS

Therefore, it is possible that under alcohol intoxication hemoglobin affinity with oxygen is affected not only by high level of acetaldehyde-modified hemoglobin molecules, but also by an increase in nitrosylhemoglobin. The introduction of L-arginine and induction of L-arginine/NO-system under AI lead to the formation of additional NO, with exerts a corrigent effect on the functional state of hemoglobin.

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