Oxidative Modification of Erythrocyte Membranes in the Acute Stage of Severe Craniocerebral Trauma and Its Correction with Clonidine

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Acute posttraumatic stage of severe craniocerebral trauma was characterized by considerable activation of lipid peroxidation and exhaustion of the antioxidant enzyme system. Oxidative modification of membranes was accompanied by structural and functional changes. The antihypoxic effect of clonidine was realized directly in the lipid phase of membranes. This preparation inhibited lipid peroxidation and activated antioxidant enzymes, which prevented the development of structural and functional changes in membranes.

Key Words: *craniocerebral trauma; lipid peroxidation; antioxidant system; oxidative stress; clonidine*

Pharmacological approaches to the therapy of craniocerebral traumas (CCT) are determined by the mechanisms of damage to neural cells, *e.g.* disturbances in cell homeostasis due to toxic effect of excitatory amino acids and modification of cell membranes during oxidative stress. Much attention was given to clonidine as a promising neuroprotector. Clonidine produces a variety of pharmacological effects that are probably realized via the nonspecific mechanism during its interaction with membrane structures. Membranotropism of clonidine can be related to high lipophilic activity [3]. To evaluate whether nonspecific membranotropic interaction contributes to the effect of clonidine, we studied the effects of this preparation on lipid peroxidation (LPO) and structural and functional properties of erythrocytes, *i.e.* the cells lacking receptors and not depending on expression of nuclear genes and activity of mitochondria.

We studied the intensity of LPO and activity of the antioxidant system (AOS) in membranes of erythrocytes from patients with severe CCT receiving standard drug therapy plus clonidine.

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MATERIALS AND METHODS

Tweenty patients with severe CCT (13 men and 7 women, average age 34.9±1.7 years) were examined. Group 1 patients (n=10) received standard drug therapy. Combination therapy in group 2 patients (n=10) included standard drugs and clonidine (Organika). Clonidine in a daily dose of 225 mg was given for 7 days after trauma at 8-h intervals. The control group included 15 age- and sex-matched healthy donors.

The state of patients was determined as described elsewhere [5]. The degree of wakefulness was determined [15]. The amount of malonic dialdehyde (MDA) in erythrocyte membranes was measured by the method of I. D. Stal'naya and T. D. Gorishvili [7]. The concentration of conjugated dienes (CD) was estimated by the method of I. D. Stal'naya [7]. The content of Schiff bases (SB) was evaluated by the method of Bidlack et Dyel [7].

Membrane lipids were extracted [11]. Microviscosity of erythrocyte membranes was evaluated by the F_E/F_M ratio, where F_E and F_M are fluorescence of pyrene excimers and monomers, respectively. Maximum fluorescence for excimers and monomers was measured at 393 and 470 nm, respectively. The intensity of fluorescence was determined at excitation wave-

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length of 334 nm. Microviscosity of zones in protein-lipid contacts (annular lipids) was measured at the excitation maximum of 282 nm. The degree of protein incorporation into the lipid bilayer (ΔF) was evaluated by quenching of protein fluorescence with pyrene at the excitation maximum of 282 nm. Polarity of pyrene probe environment in membranes was determined by the F_{372}/F_{393} ratio. This parameter reflects the ratio between pyrene fluorescence in erythrocyte suspension of at the excitation maximum of 334 nm and fluorescence wavelengths of 372 and 393 nm. Polarity in the zone of annular lipids was estimated by the F_{372}/F_{393} ratio, which characterizes the ratio between pyrene fluorescence at the excitation maximum of 282 nm at the same fluorescence wavelengths. For evaluation of structural parameters the erythrocyte suspension was adjusted to optical density of 0.72 U at absorption wavelength of 650 nm. The final concentration of pyrene was 8 μM. Fluorescence spectra were recorded on a Hitachi-650-60 spectrofluorometer [2]. Superoxide dismutase (SOD) activity in erythrocytes was measured as described elsewhere [12]. Hemoglobin was removed from the hemolysate and plasma by the method of Minami et Yoshikama. Catalase activity in erythrocyte lysate was estimated by the method of M. Luck [7]. The content of extraerythrocytic hemoglobin (EEH) was evaluated by the standard anmethemoglobin method using Reanal kits [7].

RESULTS

The intensity of LPO sharply increased in group 1 patients on day 1 of the posttraumatic period. These changes were accompanied by an increase in activities of antioxidant enzymes SOD and catalase on days 1-3 (Table 1).

The intensity of LPO tended to increase in the follow-up period. The amount of LPO products reached maximum on days 3-7 after trauma. In this period activities of SOD and catalase were lower than 1 day after trauma, which reflects ischemia of organs and tissues. Considerable activation of LPO was accompanied by changes in structural and functional properties of erythrocyte membranes. The F_E/F_M ratio at 334 nm characterizing the state of membrane lipid bilayer was below normal at various terms after trauma. We revealed an increase in relative microviscosity of membrane lipids, changes in viscoelastic properties of the membrane, and increase in cell rigidity. The observed changes reflect the decrease or loss of erythrocyte deformity. This process is followed by a loss of microcirculatory properties, decrease in the resistance of erythrocytes, and increase in the degree of hemolysis. These data are confirmed by changes in the amount of EEH, which 5.9-fold surpassed normal on day 7 ($p<0.001$).

The F_E/F_M ratio at 282 nm characterizing the state of annular lipids was below normal at various terms after trauma. Pathological changes in these zones impair not only physicochemical properties of membranes, but also oxygen-transporting function of erythrocytes [8]. ΔF progressively decreased to 30.9-37.6% of normal on days 3-7. The observed changes reflect displacement of proteins from the lipid matrix of membranes and dysfunction of integral proteins (*e.g.*, receptor centers and ion channels). Environmental polarity of the pyrene probe in the lipid layer (F_{372}/F_{393}, 334 nm) and zone of annular lipids (F_{372}/F_{393}, 282 nm) markedly increased at various terms after trauma. These

TABLE 1. LPO, AOS, and Structural and Functional Properties of Erythrocyte Membranes in Group 1 Patients

Parameters	Normal	Stage		
		Day 1	Day 3	Day 7
MDA, nmol/mg lipids	1.75±0.43	4.48±0.92***	8.97±0.95*	10.49±1.24*
CD, nmol/mg lipids	6.63±0.49	12.57±1.13**	15.39±0.86*	17.97±1.42*
SB, rel. U/mg lipids	2.28±0.26	5.93±0.44*	10.56±0.53*	13.05±0.63*
Catalase, nmol/H_2O_2/ml/mg/min	73.08±3.42	127.35±1.15*	75.34±1.26****	63.13±1.38****
SOD, μmol/mg lipids	3.19±0.24	6.90±0.42*	3.85±0.30****	2.16±0.39****
F_E/F_M (344)	0.78±0.09	0.51±0.08****	0.43±0.06**	0.38±0.04**
F_E/F_M (282)	3.66±0.28	2.18±0.34**	1.85±0.11*	1.64±0.06*
$F_{372/393}$ (334)	1.02±0.01	1.24±0.04*	1.35±0.05*	1.42±0.04*
F_{372}/F_{393} (282)	1.15±0.05	1.46±0.06*	1.57±0.05*	1.68±0.07*
ΔF	1.65±0.05	0.85±0.05*	0.62±0.07*	0.51±0.06*
EEH, μmol/liter	1.46±0.45	4.49±0.41**	7.90±0.86**	8.16±0.92**

Note. *$p<0.001$, **$p<0.01$, ***$p<0.02$, and ****$p<0.05$ compared to normal.

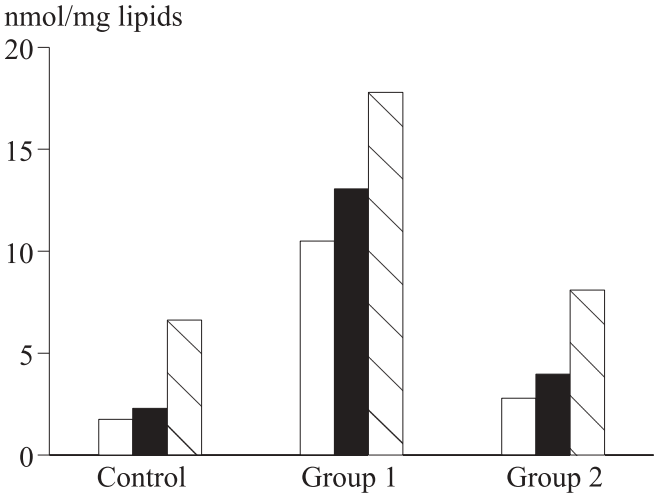

Fig. 1. LPO in erythrocyte membranes from patients on day 7 after trauma. Light bars: MDA. Dark bars: Schiff bases. Shaded bars: conjugated dienes.

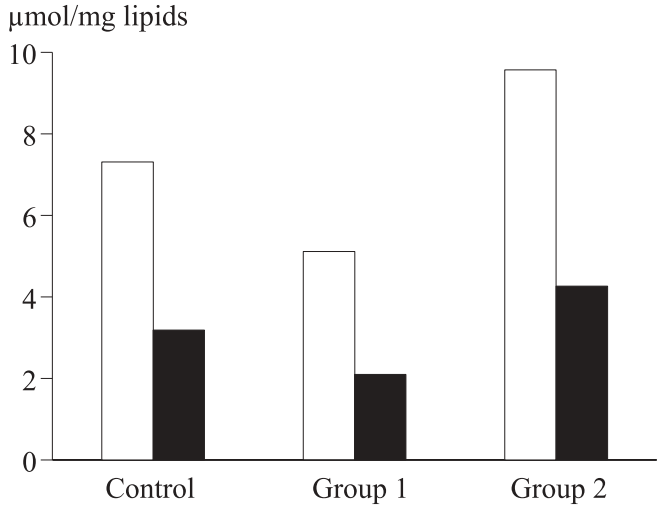

Fig. 2. Antioxidant enzymes in erythrocyte membranes from patients on day 7 after trauma. Light bars: catalase. Dark bars: SOD.

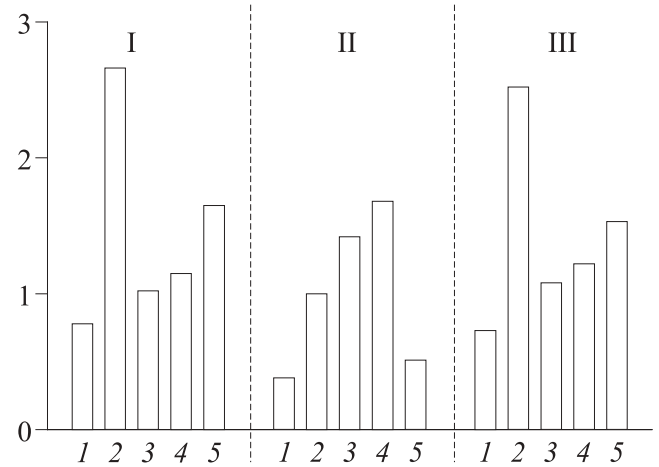

Fig. 3. Structural and functional properties of erythrocyte membranes from patients on day 7 after trauma. F_E/F_M (344, *1*), F_E/F_M (282, *2*), F_{372}/F_{393} (334, *3*), F_{372}/F_{393} (282, *4*), ΔF (*5*). Control (*I*), group 1 (*II*), and group 2 (*III*).

changes reflect decreased fluidity and increased rigidity of membranes and appearance of hydrophilic clusters, which aggravates instability of the membrane and increases its permeability [3].

Our results show that severe bioenergetic hypoxia developed over the first hours after CCT, which manifested in LPO activation and increased permeability of cell membrane [6].

The intensity of LPO increased less significantly in group 2 patients 1 day after trauma. On day 7 the contents of CD, MDA, and SB in these patients were lower than in group 1 patients by 2.3, 4.4, and 3.9 times, respectively ($p<0.001$, Fig. 1).

Inactivation of membrane prooxidants was accompanied by activation of antioxidant enzymes catalase and SOD at various terms after trauma. It should be emphasized that activity of enzymes in group 2 patients was higher than in group 1 patients (Fig. 2).

Antihypoxic protection of the brain with clonidine stimulated AOS in erythrocyte membranes, inhibited LPO, and maintained activities of antioxidant enzymes at a stationary level. These changes improved structural and functional properties of membranes. We observed a slight increase in membrane rigidity and microviscosity of annular lipids on day 1 of the posttraumatic period (Fig. 3). Statistically significant differences from group 1 patients were found 3-7 days after trauma ($p<0.05$). Therefore, clonidine normalized lipid-lipid and protein-lipid interactions in the membrane. ΔF underwent similar changes, which reflects stabilization of cell membranes and integral proteins in the lipid matrix. In group 2 patients the F_{372}/F_{393} (334 nm) and F_{372}/F_{393} ratios (282 nm) considerably increased compared to those in group 1 patients. This reflects the decrease in membrane permeability and their stabilization.

EEH content in group 2 patients increased less significantly than in group 1 patients, which was related to suppression of LPO. EEH concentration in group 2 patients was 32% lower than in group 1 patients ($p<0.02$). On day 7 after trauma EEH content in group 2 patients was 2.7 times lower than in group 1 ($p<0.01$). Thus, the degree of membrane destruction decreased in group 2 patients. The decrease in EEH concentration abolished metabolic changes [9], since the appearance of free hemoglobin in the plasma was accompanied by impairment of microcirculation and thrombocytopenia. EEH interacts with serotonin receptors on platelets and smooth muscle cells in the microcirculatory bed, leads to platelet destruction, induces spasm in the system of microcirculation, and aggravates symptoms of circulatory hypoxia [10].

Our results indicate that combination therapy with clonidine modulates reconstruction of the membrane and membrane proteins. Clonidine having phenyl and

imidazoline groups directly interacts with reactive oxygen species, neutralizes them and, therefore, inhibits free radical processes. The preparation activates intracellular SOD, which acts as the key enzyme of primary antioxidant protection. It can be related to reactivation of the active center in SOD that contains imidazoline groups (similarly to clonidine). Clonidine produces the membranotropic and membrane-stabilizing effects, which is associated with high lipotropic activity [13], accumulation in the lipid bilayer of membranes, and inhibition of LPO in the membrane lipid phase. Clonidine prevents or reduces glutamatergic neurotoxicity and blocks cascade pathological reactions that include Ca^{2+} paradox, hyperactivation of neuronal and inducible nitric oxide synthases (nNOS and iNOS), overproduction of $NO^{\bullet}$, and generation of cytotoxic reactive oxygen metabolites. Clonidine has the imidazoline ring and probably inhibits the non-Fenton mechanism of hydroxyl radical generation, which is characterized by highest toxicity and serves as a strong activator of LPO. Published data show that this mechanism of hydroxyl radical generation plays an important role in brain tissues [1].

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