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Regular paper

Oxidized proteins and activity of the Cl^-/HCO_3^- exchanger in erythrocytes of patients with acute alcohol intoxication

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Background: The purpose of this research was to study the morphological properties and the products of oxidative protein modification in erythrocytes of patients with acute alcohol intoxication. Two groups of subjects were analyzed. The first one included 39 patients with acute alcohol intoxication. The second group consisted of 14 healthy subjects. Methods: In erythrocytes the activity of Cl^-/HCO_3^- exchanger, the reactive protein carbonyl derivatives and membrane-bound hemoglobin concentration were measured. Results: Our results demonstrated strong alteration of the Cl^-/HCO_3^- exchanger activity in erythrocytes of patients with acute alcohol intoxication. A delay in the beginning of hemolysis during incubation of erythrocytes in the ammonium medium was observed. The concentration of protein carbonyls in erythrocytes of patients significantly increased in comparison to the control ones. A decrease in the membrane-bounded hemoglobin was observed as well. Conclusions: These findings indicate that ethanol toxicity is manifested by alteration of oxidized protein concentration and Cl^-/HCO_3^- exchanger activity in erythrocytes. It is hypothesized that oxidized proteins are implicated in modulation of the erythrocyte cell volume regulation.

Key words: oxidized proteins; Cl^-/HCO_3^- exchanger; erythrocytes; acute alcohol intoxication

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Abbreviations: AAI, acute alcohol intoxication; MCV, mean corpuscular volume; MBHb, membrane-bound hemoglobin; RBC, red blood cells; RPCD, reactive protein carbonyl derivatives

INTRODUCTION

Acute alcohol intoxication (AAI) is a clinically harmful condition that commonly follows the ingestion of a large amount of alcohol (Dolganiuc & Szabo, 2009). AAI can induce acute alcoholic hepatitis and cause various negative cardiovascular and gastrointestinal effects. AAI-related metabolic alterations include hypoglycemia, lactic acidosis, hypoalbuminemia, hypokalemia, hypocalcemia, and hypophosphatemia. Respiratory depression is one of the life-threatening complications of AAI, as the alcohol-intoxicated subjects are at risk of hypoxemia (Vonghia *et al.*, 2008). After penetration into erythrocytes, ethanol can be converted to acetaldehyde, the process in which catalase is involved (Tyulina *et al.*, 2000). Intracellular acetaldehyde has the ability to generate free radical species and is also involved in the formation of glycation end products and protein modification. These processes are known to have serious deleterious effects. Thus, alcohol and its metabolites have direct and indirect effects on the properties and functions of blood cells. Direct consequences of excessive chronic alcohol consumption also include toxic effects on the formation and growth of red blood cells (RBCs), white blood cells and platelets. Indirect effects of alcohol are associated with numerous disorders of blood cell functions (Ballard, 1997; Heermans, 1998). *In vitro*, alcohol induces a decrease in erythrocyte shape formation, thus increasing dynamic membrane fluctuations (Gurtovenko & Anwar; 2009; Lee *et al.*, 2015). Several *in-vitro* studies have shown ethanol-induced alteration of the 'Mean Corpuscular Hemoglobin Concentration' and erythrocyte 'Mean Cell Volume" (Fehr *et al.*, 2008; Tyulina *et al.*, 2002).

These results demonstrate that medical research was focused on the mechanisms of chronic alcohol abuse or *in-vitro* studies of ethanol-induced effects. Incidentally, the mechanical and biochemical infringements in erythrocytes in AAI patients have not been clarified yet. Detailed knowledge of the mechanisms of alterations in RBC morphological properties and the related biochemical parameters will facilitate to better understand their role in AAI development and progress.

The purpose of the present investigation was to study morphological properties of erythrocytes and the products of oxidative protein modification in the blood of AAI patients. The products of oxidative protein modification, the reactive protein carbonyl derivatives, and membrane-bound hemoglobin in erythrocytes were measured. Reactive protein carbonyl derivatives (RPCD) are the most stable products of the protein oxidative modification. The accumulation of RPCD in cells and tissues is considered a deleterious factor (Semchyshyn, 2014). Membrane-bound hemoglobin is also considered to be a variant of the modified protein. The functions of the membrane-bound hemoglobin are not clear yet. Rifkind and Nagababu (Rifkind & Nagababu, 2013) assumed that under hypoxia the proportion of membrane-bound hemoglobin increases. It leads to structural aberrations of erythrocyte membranes, disruption of erythrocyte deformability, affects calcium and potassium transport.

MATERIALS AND METHODS

Patients and ethics. Two groups of subjects were analyzed. The first one included 39 patients aged from 22 to 60 with acute alcohol intoxication admitted for

hospitalization in the toxicological department of the Regional Medical Center in Karaganda city. The second group consisted of 14 healthy subjects of the same age group. This investigation was approved by the ethics commission at Karaganda State Medical University. All patients and healthy subjects had received full information on possible inconveniences and complications of the blood sampling before giving their written informed consent.

All patients had no history of excessive alcohol abuse prior to the research. To support the acute alcohol intoxication diagnosis we used "The toxic effect of alcohol (adults and children)" protocol recommended by the Expert Council "Republican Center for Health Development" of the Ministry of Health and Social Development of the Republic of Kazakhstan (30.10.2015) and International Classification of Diseases (ICD-10-WHO Version, 2016). The diagnosis verification was based on the medical history and the results of an objective examination based on "The toxic effect of alcohol (adults and children)" protocol. The blood alcohol concentration in patients with AAI amounted in average to 2.68 ppm. Medical history included the type and quantity of the liquor consumed and the duration of the symptoms. It should be noted that at times the medical history was difficult to obtain.

Among the AAI patients there were 75% of males and 25% of females, 81% of them being within the working-age category (aged 30–60). The largest number of AAI patients was recorded in the age group of 50 to 59 years (36%). the lowest number was in the age group of 60 years and above (5.95%) and 13% of AAI patients were recorded under the age of 30. The exclusion criteria were: age below 18 years and above 60 years, pregnancy, other substance-related intoxications (alcohol other than ethanol), severe autoimmune diseases, anemia, heart attacks, strokes, pathology of the hematopoietic system.

Biochemical blood tests. The time lapse between drinking episode and blood sample collection was no more than 6 hours. Blood was stabilized by heparin. Plasma was separated from erythrocytes by centrifugation. All blood tests were conducted within one hour after the blood collection.

Assay of the Cl^-/HCO_3^- exchanger. This was performed using the protocol of Mindukshev and others (Mindukshev *et al.,* 2010). One hundred μL of blood was placed in isotonic solution in which sodium ions had been replaced by ammonium ions (140 mM NH_4Cl, 5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$). All reagents used were produced by JSC "Kupavnareactiv", Russia, Moscow region, Old Kupavna, Kirov str. 29. Under these conditions, the alkalization of intracellular pH by the penetration of NH^{4+} led to the activation of the surveyed exchanger, regulating entrance of chloride anions, which led to swelling of the cells. The cell volume changes (Mean Corpuscular Volume, MCV, fL) were recorded in a hematology analyzer BC-3200 (Mindray, Shenzhen, China) during 10 min incubation in the ammonium medium. The time of the sharp decrease in the erythrocyte volume was noted. This time was considered as the onset of the erythrocyte hemolysis. We included two extra ratios into the results: the MCV change (ΔV) and the velocity of ΔV change (υΔV, fL/min) observed during 10 min incubation in the ammonium medium.

Assay of reactive protein carbonyl derivatives and membrane-bound hemoglobin. The concentration of reactive protein carbonyl derivatives was determined following the protocol of Levine and others (Levine *et al.,* 1990). Membrane-bound hemoglobin (MBHb) was detected following the protocol of Toktamysova and Birzhanova (Toktamysova & Birzhanova, 1990). These measurements were performed using a UV-IS spectrophotometer Model PD-303UV.

Statistical analyses. This was performed using the non-parametric Mann-Whitney U-test (for independent variables).

RESULTS

Table 1 shows the results of the study of RBC hemolysis obtained for the control subjects and AAI patients. The results demonstrate that 13% of the control

Table 1. Hemolysis of erythrocytes of patients with acute alcohol intoxication

	Incubation time in the ammonium medium (min)	Percentage of non-hemolysed cells (%)	95% confidence interval
Healthy subjects (control)	1	100	
	2	100	
	3	87	60–98
	4	53	27–79
	5	7	0.2–32
	6	0 (full hemolysis)	
Patients with acute alcohol intoxication	1	100	
	2	94	80–99
	3	85	68–95
	4	73	54–87
	5	39	23–59
	6	21	9–39
	7	15	5–32
	8	9	2–24
	9	3	0.1–16
	10	0 (full hemolysis)	

Table 2. MCV, ΔV MCV and ÚMCV in patients with acute alcohol intoxication, mean values ± S.D. MCV, mean corpuscular volume; ΔMCV, the MCV change; ÚMCV, the velocity of ΔMCV change observed during 10 min of erythrocyte incubation in the ammonium medium.

Groups	MCV max (fL)	ΔMCV (fL)	ÚMCV (fL/min)
Healthy subjects	100.06±5.42	10.34±2.75	2.36±0.63
Patients with acute alcohol intoxication	108.33*±7.25	13.64*±4.94	2.71±0.93
p-level (Mann-Whitney U Test)	0.0002	0.0046	0.2757

*Marked tests are significant at $p<0.05$

Table 3. Oxidized proteins in erythrocytes of patients with acute alcohol intoxication, mean values ± S.D. RPCD, reactive protein carbonyl derivatives; MBHb, membrane-bound hemoglobin.

Groups	RPCD (nmoles/ml)	MBHb (%)
Healthy subjects	9.62±2.22	9.53±2.84
Patients with acute alcohol intoxication	17.92*±3.54	7.46*±2.44
p-level (Mann-Whitney U Test)	0.0000	0.0197

*Significant at $p<0.05$

subjects' RBCs were hemolyzed between the 3rd and the 4th minute of incubation in the ammonium medium. Full hemolysis was observed after the sixth minute of incubation in the ammonium medium.

Different results were observed for AAI patients. First of all, RBC hemolysis started earlier (between the 1st and the 2nd minutes of incubation in the ammonium medium). The time required for all erythrocytes of AAI patients to undergo hemolysis was increased to 10 min as compared to 6 min for the control subjects.

MCV and ΔMCV ratios in AAI patients were significantly higher than those for the control subjects (Table 2).

These results demonstrate strong alteration of the Cl^-/HCO_3^- exchanger activity in erythrocytes of AAI patients leading to change in RBC volume regulation. We also observed a delay in the beginning of the hemolysis during incubation of RBCs in the ammonium medium. At the same time the concentration of reactive protein carbonyl derivatives in RBCs of AAI patients significantly increased as compared to the control subjects. A decrease in the membrane-bound hemoglobin in RBCs of AAI patients was observed as well (Table 3).

These results demonstrate divergent trends in the oxidized intracellular proteins alteration of AAI patients' RBCs.

DISCUSSION

The results obtained earlier by other authors showed a change in the deformability and an increase in the sphericity of erythrocytes *in vitro* under the ethanol effect (Lee *et al.*, 2015; Sonmez *et al.*, 2013). Ethanol exerted toxic effects by production of reactive oxygen species and inducing lipid peroxidation in various tissues and cells (Hosseini *et al.*, 2017). Our results showed an alteration of the morphological properties and several biochemical parameters of erythrocytes in AAI patients. We observed an increase in MCV, alteration of erythrocyte volume regulation, presence of the subpopulations of RBCs with an elongated period of hemolysis during incubation in the ammonium medium. This increase was dependent on the alteration of the Cl^-/HCO_3^- exchanger activity. Synchronously, in AAI patients' erythrocytes a significant increase in the reactive carbonyl protein products was observed. We surmised that cytoskeletal proteins and hemoglobin could be the most likely substrates for the formation of reactive carbonyl protein products. Cytoskeletal proteins were connected with membrane–bound proteins, including the Cl^-/HCO_3^- exchangers (Bruce *et al.*, 2003). Oxidative infringement of cytoskeletal proteins and accumulation of reactive carbonyl protein products affected Cl^-/HCO_3^- exchanger activity and led to an increase in the erythrocyte volume. These infringements were caused by an indirect effect of acetaldehyde and ethanol (*via* generation of free radical species) and a direct effect of acetaldehyde on erythrocyte protein modification. Under oxidative stress, cytoskeletal proteins can be aggregated with hemoglobin (Olszewska *et al.*, 2012), inducing an alteration of gas exchange in RBCs.

It is believed that ion transport in RBCs is regulated by O_2 tension (Stefanovic *et al.,* 2013). We hypothesize that under condition of respiratory depression, as one of AAI complications, an increase in the RBC volume could be a compensatory mechanism for ion transport regulation. In this case, a decrease in the membrane binding of hemoglobin in erythrocytes of AAI patients could be responsible for a change in transport protein activity, thus affecting cell volume regulation. On the other hand, oxidative damage of the RBC proteins induced profound metabolic disorders leading to the development of hypoxia and alcoholic anemia. Further studies will be necessary to determine the Cl^-/HCO_3^- exchanger activity and other biochemical alterations in AAI patients' RBCs depending on the stages and complications of the acute alcohol intoxication.

Taken together, our findings show that ethanol intoxication resulting from acute alcohol consumption is manifested by alteration of oxidized protein concentration and Cl^-/HCO_3^- exchanger activity in erythrocytes of affected individuals. We hypothesize that oxidized proteins are implicated in modulation erythrocyte cell volume regulation.

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