Original Article

Antioxidant system and structure of erythrocytes in people with psychological and behavioural disorders due to alcohol abuse

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Abstract

The pathogenesis of illness associated with alcohol abuse plays an important role not so much in the duration of alcoholic beverages consumption by patients, as in the oxidative stress in different cells. Erythrocytes of peripheral blood are considered to be passive ‘informative cells’, and patients with psychological and behavioral disorders due to alcohol abuse have not been studied sufficiently. One of the tasks of this work was to find out through which component the activity of the antioxidant system and the structural state of erythrocytes in people with psychological and behavioral disorders due to alcohol abuse changes. It has been shown that in people with psychological and behavioral disorders due to alcohol abuse oxidative stress in peripheral blood erythrocytes increases, which is manifested by an increase in the concentration of H₂O₂ and organic hydroperoxides, as well as a decrease in the ratio of glutathione / glutathione disulfide and glutathione transferase activity. Increasing the size of peripheral blood erythrocytes against the background of decreased activity of glutathione peroxidase can serve as a reliable marker for alcohol addiction, and changes in other indicators of oxidative stress reflect non-specific violations, probably from hepatocytes responsible for saturation of peripheral blood erythrocytes with appropriate antioxidants.

Keywords: erythrocytes, oxidative stress, antioxidants.

Introduction

Alcohol abuse (AI) is a fairly widespread phenomenon among people of different ages in many countries of the world (Goffman, & Orlova, 2012; Blagovskaya, & Kinova, 2017). According to Gorkova (2018) in people with AI addiction a special nosological unit develops, namely, a bio-psycho-social disease, in which all signs of a rapidly progressive neurodegenerative disease are often observed, which is clinically manifested in the gradual loss of memory and mental abilities (Polyakova, & Yusupova, 2017) up to AI-addiction dementia (Vostrikov, Zelentsov, Maiorova, Vostrikov, Pavlenko, & Shabanov, 2008).

Recent studies confirm the most common pattern of the pathogenesis of this disease as a multifactorial disease that combines genetic, biochemical and physiological concepts (Rosly, Abramov, Vodolazhskaia, & Shuliak, 2005). Meanwhile a diagnosis is based on modern objective methods, among which, along with well-known clinical and psychological methods of diagnosis, an important place is occupied by instrumental, immuno-enzymatic and biochemical methods of diagnosis with the detection of alanine and aspartate aminotransferase (ALT, AST); alcohol and aldehyde dehydrogenase (ADH, AIDG) and gamma-glutamyltransferase (GGT).

Currently, the method of studying biological markers for the use of ethanol has become widely popular. Among them, the most important is the marker, which depends on the physicochemical properties of ethanol and acetaldehyde, which is an increase in the average corpuscular erythrocyte volume (ACEV). Along with this indicator, other indicators are also being studied, they are the deterioration of erythrocyte filtering (Er); the presence of thrombocytopenia, an increased activity of ADH and microsomal ethanol oxide system, a decrease of AIDG activity and determination of acetal aldehyde level in blood.

Nevertheless, there is a large number of methods of treatment and physical therapy for people with psychological and behavioural disorders due to alcohol abuse (Zagorskaya, & Nazarkina, 2016). However, they are quite problematic and uncertain, in terms of favourable outlook of long-term results.

According to the biochemical hypothesis, the accumulation of biological markers for the use of ethanol in tissues initiates a cascade of reactions, which leads to complex pathology and clinical manifestations of the disease (Rosily, Abramov, Agaronov, Ivanov, & Shulyak, 2004). Complexes of proteins and their soluble oligomers break apart under the influence of toxic products of AI transformation (“fatal synthesis”) and stimulate

the production of active oxygen-containing metabolites (AOM), indicating the connection between the use of AI, oxidative stress and destruction of various cells of the body (Rosly, Abramov, Agaronov, Shipiko, & Shulyak, 2004; Blagovskaya, & Kinova, 2017).

Many years of research have shown that people with psychological and behavioural disorders due to alcohol abuse have anomalies of oxidative processes in the brain (Rosly, Abramov, Akhmetov, Ryneiskaia, Rykhletsnyi, & Shuliak, 2004), as well as in other tissues and cells (Rosly, Abramov, Shipiko, Rozhkova, & Shulyak, 2005), including peripheral blood Er (Bohan, & Prokopieva, 2004).

In the pathogenesis of AI addiction, an important role is given to oxidative stress in the nerve cells. In this case, Er are considered as passive 'informative cells', reflecting the exquisite reaction (Popel', 1994) on biochemical processes occurring in the whole organism (Kushnerova, 2010). However, the exquisite reactions of Er in people with psychological and behavioural disorders due to alcohol abuse have hardly been studied, there is insufficient information on the oxidative stress in Er in these patients, and the available data are extremely controversial. Until now, the concentrations of organic hydroperoxides and H_2O_2 in Er and their changes in the dynamics of the disease and during the treatment of AI addiction, as well as the associated level of rehabilitation potential (RP) in such patients, and the ambiguous results of measurement of intracellular concentrations of glutathione (GSH) and glutathione disulfide (GSSG) are still unknown.

Recently, we have found out that AI causes lysis of intact Er in humans in vitro, which is amplified by the action of the inhibitor of GLP, which is one of the antioxidant enzymes. This allows suggesting that the Er-toxicity of AI both in vitro and in vivo depends on the state of the antioxidant system (AOS) Er (Skripka, Safiullina, & Abushaieva, 2006). Disruption of AOS is naturally reflected in the structural features of Er, which was shown in a variety of pathological processes (Rosly, Abramov, Vodolazhskaia, & Shuliak, 2005).

Therefore, it is interesting to observe the comparative study of the biochemical properties of AOS and structural changes of Er in AI-addicted, practically healthy people and people with psychological and behavioural disorders due to alcohol abuse, taking into account the level of their RP.

The aim of the research: To find out how the antioxidant status and structure of erythrocytes changes in people with psychological and behavioural disorders due to alcohol abuse, depending on their level of rehabilitation potential.

The methods and the organization of the research.

The research was carried out at the rehabilitation center for people with psychological and behavioural disorders due to alcohol abuse (Ivano-Frankivsk, Ukraine).

The study involved 60 male patients aged 25-40 years an average of $32,5 \pm 2,11$ years old, who gave their (or close relatives') consent in writing took part in the study. The inclusion of the patients in the study was based on the stability of the body mass index and the data of clinical analysis of blood. For various reasons, 12 previously-involved patients were subsequently excluded from the study. Diagnosis: psychological and behavioural disorders due to alcohol abuse were based on the criteria of the Consortium for the development of diagnostic criteria for AI addiction (Goffman, Meliksetian, & Orlova, 2010; Petrova, 2015) and verified by the Bureau of Medical and Social Expertise at Ivano-Frankivsk Psychoneurological Dispensary.

The following groups were formed to study the activity of AOS and the restructuring of Er: the main group (MG-1) – 12 patients with psychological and behavioural disorders due to alcohol abuse, who had a high level of RP; the main group (MG-2) is 12 people with psychological and behavioural disorders due to alcohol abuse with an average level of RP; the main group (MG-3) – 12 patients with psychological and behavioural disorders due to alcohol abuse with a low level of RP; the control group (CG) – 12 practically healthy volunteers, selected according to the criterion for the absence of a history of clinical diseases, neurological or psychiatric disorders or appropriate therapy.

Venous blood was taken in the morning on an empty stomach. Laboratory blood test was carried out in the first 30 minutes after blood taking in the rehabilitation center, and preparation of Er for scanning electron microscopy (SEM) and biochemical analysis – in the clinical laboratory of Central City Hospital in Ivano-Frankivsk.

Er products for SEM were prepared according to the generally accepted method (Ebner, Schillers, & Hinterdorfer, 2011) and investigated using a scanning electron microscope JEOL-25A-T225 (Japan) with an accelerating voltage of 20 kV, and the average corpuscular volume of the erythrocyte (ACVE) was determined by the morphometric ImageJ program (Vostrikov, Zelentsov, Maiorova, Vostrikov, Pavlenko, & Shabanov, 2008).

The state of the AOS Er was evaluated by measuring the activity of superoxide dismutase (SOD) (CF 1.15.1.1), GLP (KF 1.11.1.9), catalase (CT) (CF 1.11.1.6), GLR (CF 1.8.1.7), GLT (CF 2.5. 1.18) and glucose-6-dehydrogenase (G6FDG) (CF 1.1.1.49), concentrations of GSSG, GSH, H_2O_2 and organic hydroperoxides.

Extraction of Er, preparation of hemolysate and determination of activity of SOD, GLP, CT, and GLR were performed according to the methods described earlier (Kaminsky, Suslikov, & Kosenko, 2010; Kosenko, Suslikov, Venediktova, & Kaminsky, 2010). The activity of G6FDG was determined spectrophotometrically (William, Valentine, & Paglia, 1991). The GLT activity was measured at 340 nm with 1-chloro-2,4-dinitrobenzene as a substrate, as described by Kozer et al., (2003). The unit of the enzyme activity was the amount of enzyme that catalyzed the formation of 1 micromol of GSH / 1-chloro-2,4-dinitrobenzene complex for 1 min per 1 ml of hemolysate.

To determine the intracellular concentrations of metabolites, acidic extracts of Er were prepared. 0.2 ml of cells were mixed with 1 ml of cold ($-20^{\circ}C$) mixture of 6% HCl_4 / 40% C_2H_5OH and centrifuged at 10000 g and $-10^{\circ}C$ for 1 min. The supernatant was neutralized with 30% KOH ($-20^{\circ}C$). The sediment was removed by centrifugation, and a transparent supernatant was used to determine the concentrations of metabolites.

H_2O_2 and organic hydroperoxides were determined by the method described by Mason (2013). 0.5 ml of the extract was incubated at $25^{\circ}C$ for 5 minutes in 0.5 ml of the buffer solution containing 0.12 M Tris, pH 7.6, 0.2 mM EDTA and 200 units / ml of CT. Then 0.2 mM NADPH, 10 μ l of purified GLP (activity 2.5 μ mol / min per 1 ml) and 0.5 mM GSH were added. After 10 min incubation at $37^{\circ}C$, light absorption at 340 nm was recorded. 0.5 ml / ml of GLR was then added, incubated for 10 min, and absorbance was recorded at 340 nm. The amount of oxidized NADPH and the intracellular concentration of organic hydroperoxides in Er was calculated by the absorption difference after the first and second measurements.

Similarly, the total concentration of peroxides (organic hydroperoxides and H_2O_2) was determined by performing the same procedures in the absence of CT. By the difference between the total peroxide concentration and the concentration of organic hydroperoxides, the intracellular concentration of H_2O_2 , while GSH was calculated by the method based on the non-enzymatic oxidation of GSH 5,5'-dithiobis-2-nitrobenzoate (DTNB) with the formation of oxidized glutathione (GSSG) and 5-thio -2-nitrobenzoate (TNB) and TNB absorption recording at 412 nm. 20 μ l of Er suspensions were mixed with 100 μ l of cooled 10 mM HCl to fix endogenous glutathione. Cell membranes were destroyed by a 3-fold freeze-thaw cycle.

The sample was centrifuged at 14000 g and $4^{\circ}C$ for 5 minutes, supernatant proteins were precipitated by adding 50 μ l of cooled 10% sulphosalicylic acid. The sediment was removed by centrifugation, and a transparent supernatant was used to determine the total concentration of GSH $\pm$ GSSG. The analysis was performed at $30^{\circ}C$ in 1 ml of medium containing 100 mM Na_2HPO_4 , pH 7.5, 4.5 mM of Na_2EDTA , 2 mM of NADPH, 0.6 mM of DTNB, 0.84 μ mol / min of GLR. The reaction was started by adding 10-20 μ l of Er extract. For calculations, the absorption coefficient of TNB, equal to $\epsilon_{412} = 13.6 \text{ mM}^{-1} \bullet \text{cm}^{-1}$, was used.

To determine GSSG, 2 μ l of 2-vinyl pyridine (for endogenous GSH binding) were added to 100 μ l of the protein-free supernatant, the mixture was neutralized with 30% $KHCO_3$, incubated for 60 minutes at $25^{\circ}C$, and centrifuged. In the supernatant, GSSG concentration was determined. 925 μ l of buffer solution (100 mM of Na_2HPO_4 , pH 7.4, 4.5 mM of Na_2EDTA), 0.6 mM of DTNB, 0.2 mM of NADPH were added to the spectrophotometer dish. The reaction was started by adding 10 μ l (0.5 μ mol / min) of GLR.

The concentration of GSH was calculated by deducting the measured concentration of GSSG with the total concentration of GSH $\pm$ GSSG.

The statistical analysis was performed by using the GraphPad Prism computer program, version 4.03, for Windows ('GraphPad Software', USA). The results were expressed as the mean (M) and the standard error of the mean (m). The differences between the groups were analyzed by the ANOVA method, followed by the calculation of the Student's t-criterion for determining the statistical significance.

Results of the research and their discussion.

The values of SOD, catalase, GLR, GLP, GLT and G6FDG in ER activity in patients are shown in Figure 1 and in Table 1. Since we have not revealed sex differences in the activity of the Er enzymes, all the data presented below and hereafter are averages for individuals of both sexes included in each group.

Compared to CG, individuals from Group I have a significantly lower activity of AOS in Er, including SOD (by 26.4%), CT (by 13.8%), GLP (by 22.5%) and GLT (by 21.3%).

Fig. 1. Activity of superoxide dismutase (A), glutathione peroxidase (B), total concentration of peroxides (H₂O₂ ± organic hydroperoxides) (C) and GSH / GSSG (D) ratio in erythrocytes in the control group (CG) and depending on the rehabilitation potential: MG-1 – high, MG-2 – average, MG-3 – low. Notes: * - $p < 0,05$ ** - $p < 0,001$ when compared with the group CG; ♦ - $p < 0,05$ in comparison with MG-1.

Only the activity of GLR and G6FDH in Er does not change, depending on the level of RP of the observed.

By the RP level, patients do not differ in their activity of CT, GLR or GLT. Only the activity of GLP, G6FDG, and SOD is likely to change in MG-2 and MG-3 in comparison with the CG and MG-1 ($p < 0,05$). The activity of GLP is lower in MG-2, while the activity of G6FDG is higher in MG-2 and MG-3 compared with MG-1. On the contrary, SOD activity is probably higher ($p < 0.001$) in the CG compared to its activity in other groups, but both values are 12.9% lower than in the CG. Oxidative stress is expressed in an increase in the concentration of AKM, as shown in Figure 1C, D in Table 2.

Table 1

**Antioxidant enzymes in peripheral blood erythrocytes
in people of different age groups ($M \pm m$, $n=48$)**

Enzyme	CG	MG-1	MG-2	MG-3
CT, c-1 / ml	24,1 ± 0,9	20,7 ± 1,3*	20,0 ± 1,2*	21,4 ± 0,9*
GLR, µmol / (min • ml)	2,07 ± 0,17	1,87 ± 0,07	2,0 ± 0,14	2,11 ± 0,14
GLT, µmol / (min • ml)	1,46 ± 0,14	1,16 ± 0,0*	1,05 ± 0,10*	1,02 ± 0,14*
G6FDH, µmol / (min • ml)	3,50 ± 0,14	3,06 ± 0,20	3,60 ± 0,24 ^a	3,74 ± 0,28 ^a

Note: * - $p < 0,05$, *** - $p < 0,001$ when compared with the group of KG, ^a - $p < 0,05$ when compared with MG-1.

Table 2

**Antioxidant enzymes in peripheral blood erythrocytes
in people of different age groups (M±m, n=48)**

Indicator	CG	MG-1	MG-2	MG-3
H ₂ O ₂ , μM	0,31±0,06	0,41±0,06	0,49±0,06*	0,45±0,04*
Organic hydroperoxides, μM	0,26±0,06	0,63±0,12*	0,81±0,16**	0,93±0,2**
GSH, mM	2,10±0,06	1,85±0,1*	1,82±0,1*	1,79±0,06**
GSSG, μM	43,3±1,4	50,3±2,2*	55,7±4,9*	59,2±3,3***, ^a

Notes: * - $p < 0.05$ ** - $p < 0.01$, *** - $p < 0.001$ when compared with CG, and - $p < 0.05$ compared to KHLV.

The intracellular concentration of organic hydroperoxides in Er in MG-1 is 1.4 times higher than in CG, and then increases in MG-2 and MG-3. These dynamics correlates with an increase in the concentration of GSSG, a decrease in the GSH concentration and GSH / GSSG ratio, depending on the decrease in the RP level. Indicators in CG and MG-1 do not differ in content of H₂O₂, whereas in patients from MG-2 and MG-3, the intracellular concentration of H₂O₂ is significantly higher than in CG.

Significant differences were observed between patients with different levels of RP in Er (content of H₂O₂, organic hydroperoxides, GSH, GSSG, and GSH / GSSG ratio). In this case, the activity of GLP has a strong inverse-proportional correlation dependence on the concentration of H₂O₂ ($r = -0.972$) and the total peroxide concentration ($r = -0.854$), the activity of the GLT and the ratio of GSH / GSSG – with the total concentration of peroxides in Er ($r = -0.994$ and $r = -0.990$, respectively).

It has been established that Er in the patients from the MG have their own peculiarities which, in the first place, are manifested by an increase in a cell size (Fig. 2). Along with this, in the patients from MG-2 altered forms of Er appear inversely, and in people from MG-3 – also irreversibly altered forms of Er. Their number increases depending on the age of the patients and the duration of Al. abuse.

Fig. 2. The structure of peripheral blood erythrocytes in patients with psychological and behavioural disorders due to alcohol abuse depending on the level of rehabilitation potential: A – patients from MG-1, B – MG-2, C – patients from MG-3, compared with practically healthy people (D)

In patients with psychological and behavioural disorders due to alcohol abuse a histogram of Er distribution is asymmetric in size and multipole due to an increase in the number of variational classes of large-

size (right) wings of cellular elements (Fig. 3 A–C), in contrast to CG, where the histogram has a normal distribution (Fig. 3 D).

Fig. 3. Distribution of erythrocytes in people with psychological and behavioural disorders due to alcohol abuse, depending on the level of rehabilitation potential compared with the control group at its maximum diameter.

Comparative analysis shows that in CG the volume of the size classes in the left wing is only 7.1%, whereas in the main group of patients this percentage is gradually decreasing and ranges from 4.8% in MG–1 to 0.9% in MG–3.

Discussion

Violations in the AOS of Er in AI addictions have been described in the scientific literature, but the findings are small and ambiguous. Thus, the activity of SOD in Er in the elderly and middle-aged people with AI addiction is not different (Skripka, Safiullina, & Abushaieva, 2006) or it is below Gaponov (2016) or higher Menecier et al., (2008) than the enzyme activity in Er of young volunteers.

In our studies, the activity of SOD in Er of the people from MG–1 is significantly lower than that in CG, according to Rybka et al., (2011). The contradictory nature of the data cannot be explained without special studies or additional data not given in the quoted works, but with this contradiction, it turns out that the activity of the SOD in Er cannot serve as an AI addiction marker.

There are even less homogeneous literature data regarding the activity of SOD, GLP, and CT in Er in the patients with AI addiction. In this group of patients, the increase in SOD activity is most frequently observed (Selkoe, 1991; Kawamoto et al., 2005; Kharrazi et al., 2008), but in some cases, it does not change (Bourdel-Marchasson, 2001) or decreases (Vural et al., 2010).

The activity of the GLP with AI addiction also either increases (Anneren et al., 1986), or decreases (Vural et al., 2010), or does not change (Bourdel-Marchasson, 2001). CT activity is usually lower in people with psychological and behavioural disorders due to alcohol abuse than in healthy people (Kharrazi et al., 2008).

Such controversial data led us to carry out a more complete study – to determine the intracellular concentration of low molecular oxidants (H_2O_2 , organic hydroperoxides, and GSSG) and antioxidant one of GSH together with the activity of antioxidant enzymes in Er in all four groups of subjects.

We have shown that the activity of SOD, CT, GLP, and GLT in Er is likely to change in all patients regardless of the level of PR. The activity of CT and GLT is the same in all patients, that is, changes in the activity of these enzymes are non-specific for AI addiction. The activity of GLR does not differ in all four groups of subjects, while the activity of G6FDG decreases only in patients from MG–3.

Patients in all groups differ from those in CG with SOD and GLP activity, while there are no such differences between the people from MG–1 and MG–2. The activity of GLP correlates with the concentration of peroxides more strongly (correlation coefficient $r = -0.854$) than SOD activity ($r = -0.61$).

Thus, in this paper, it has been found out that the decreased activity of GP in Er (as compared with the activity in the group of KGV) is a marker of Al-addiction.

All low molecular indexes of oxidation stresses in Er change with Al addiction in the direction of increasing oxidative stress: intracellular concentrations of H_2O_2 , organic hydroperoxides and GSSG increase, GSH concentration and GSH / GSSG ratio decrease. Similar changes occur in the aging body, which indicates an increased loss of energy and plastic resources in patients with Al addiction (Vostrikov et al., 2008).

In the literature, there are no comparative data on the concentration of H_2O_2 and organic hydroperoxides in the Er of practically healthy people from CG and patients, depending on the RP level, and there is no sufficient information on the intracellular concentrations of GSSG and GSH. In general, our results confirm the available data of scientific literature on the increase of oxidative stress in Er with Al addiction with a low level of PR. Therefore, we conducted a structural morphometric study of the peripheral blood of patients with psychological and behavioural disorders due to alcohol abuse in order to find a correlation between the state of AOS and the degree of morphological rearrangement of the Er membrane.

It is known that as a result of biotransformation, ethanol in the body loses its toxic properties. However, the transformation of ethanol occurs in the type of 'lethal synthesis', that is, with the formation of more toxic products, creating an adverse effect on all cells of the body, in particular, having a significant influence on the production of Er, which is clinically manifested after a few days or weeks of Al abuse in large quantities.

This is most often expressed in an increase in the size of Er (their average corpuscular volume), the appearance of inversely and irreversibly altered forms, the appearance of moderately pronounced anemia (Prokopieva et al., 2005) with a shift of the histogram of cell division to the right. An increase in the number of right-wing Er histograms in all patients from the main group, regardless the level of PR, is associated with a probable ($p < 0.05$) increase in median and mode values. The above mentioned features of the histogram mean that, in response to the effect of Al, Er of small size react primarily (their number decreases sharply), while the number of macrocellular forms gradually increases, which is typical of toxic reactions that are likely to develop as a result of the release of the "first-phase stress-reaction" cells from the reserve pool of total blood circulation (Konoplya et al., 2017). As the reserve capacity of Er decreases and the patients move to a lower level of the PR, the distribution of Ep becomes more extreme: the number of intervals increases, the volumes of the individual classes vary as a result of jumps, and the achievement of the values of the central parameters is wavelike (in the CG for a monotonously rising trend). The waveform of the histogram probably reflects the unstable vibrational state of Er during the period of active intoxication of the body of the patients from the main group (Bohan, & Prokopieva, 2004). A well-known method for diagnosing Al addiction by detecting ACEV and GGT activity in the blood (Vostrikov et al., 2008). The method differs: in order to improve the accuracy of diagnosis in the early stages of the disease, the blood is taken for research in certain time intervals, the total activity of lactate dehydrogenase is being further determined in the blood serum. With a simultaneous increase of ACEV, GGT and lactate dehydrogenase alcohol addiction is diagnosed. In the presented method of diagnosis, attention is drawn to biorhythmic circadian changes in biochemical parameters of blood, but the influence of somatic disorders on them is not taken into account.

Conclusions

Strengthening of oxidative stress in Er during aging and dementia is manifested by an increase in the concentration of organic hydroperoxides and H_2O_2 , a decrease in the activity of GLT and the GSH / GSSG ratio, which have a medium and strong correlation between them.

Reduced activity of GLP with simultaneous increase of GGT and lactate dehydrogenase in peripheral blood erythrocytes, along with their structural changes in the form of a predominance of microcell forms, can serve as a new marker of Al-addiction.

Changes in other indicators of the state of the antioxidant system of peripheral blood erythrocytes are not specific for patients with psychological and behavioural disorders due to alcohol abuse.

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