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Association of Peripheral Markers of Oxidative Stress with Clinical Parameters and Inflammatory Factors in Alcoholic Patients

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Abstract—One of the fundamental problems in studying addiction is elucidation of mechanisms of alcohol dependence (AD) development. Disturbances of cellular redox balance and inflammation play an important role in AD pathogenesis. Deciphering associations between biological and clinical indicators can elucidate molecular mechanisms of disease pathogenesis. The aim of the work was to study peripheral markers of oxidative stress in patients with AD during early abstinence period and to identify their relationship with clinical parameters of the disease and inflammatory factors. In total, 84 patients with AD were studied (average age, 44.3 ± 8.2 years). The analyzed clinical parameters included patient's age, age of alcohol withdrawal syndrome (AWS) formation, disease duration, and AWS duration. The markers of oxidative stress determined in the blood plasma were oxidation products of proteins (protein carbonyls, PCs), lipids (thiobarbituric acid-reactive products, TBA-RPs), and DNA (8-hydroxy-2′-deoxyguanosine, 8-OH-dG). The content of inflammatory mediators, such as proinflammatory cytokines (IFNγ, IL-1β, IL-6, IL-8, IL-17A, TNFα) was determined in the blood serum. Blood samples of 80 conditionally healthy men (average age, 40.9 ± 9.6 years) were used as a control. Patients with AD demonstrated an increase in the content of PCs, TBA-RPs, and all analyzed cytokines (but not 8-OH-dG) compared to the control individuals. There was a direct correlation between the TBA-RP content and disease duration and inverse correlation of the PC content with the age of AWS formation and AWS duration. The content of PCs demonstrated an inverse correlation with the IL-6 concentration in the blood plasma. We also observed a positive correlation between 8-OH-dG and IL-6, TBA-RPs and IL-8, and TBA-RPs and TNFα. Therefore, the early abstinence period in patients with AD was characterized by a pronounced oxidative stress and inflammation. The obtained results expand the knowledge on the integrative contribution of oxidative stress and inflammatory factors to the AD pathogenesis and can be used in the development of new therapies.

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Keywords: oxidative stress markers, proinflammatory cytokines, alcohol dependence

INTRODUCTION

The studies of addiction have shown that similarly to many pathological conditions, alcoholism (in particular, alcohol dependence, AD) is accompanied by the development of oxidative stress. Beside general mechanisms of oxidative stress emergence, alcoholism is characterized by specific sources of free radicals,

Abbreviations: 8-OH-dG, 8-hydroxy-2'-deoxyguanosine (8-oxo-7,8-dihydro-2′-deoxyguanosine); AWS, alcohol withdrawal syndrome; AD, alcohol dependence; IL, interleukin; LPO, lipid peroxidation; IFNγ, interferon gamma; PC, protein carbonyl; TBA-RP, TBA-reactive product; TNFα, tumor necrosis factor alpha.

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including reactive oxygen species (ROS), associated with the transformation of ethanol and its main metabolite acetaldehyde in the body [1-3].

Alcohol passes through the blood-brain barrier, which causes activation of microglia and leads to the increased production of proinflammatory cytokines, development of neuroinflammation in the central nervous system, and impairment of neuroimmune communications, thus affecting behavioral responses of alcoholic patients [4-6]. Long-term alcohol consumption also weakens the intestinal barrier and promoting release into the blood of bacterial components, e.g., lipopolysaccharides (LPSs), that activate immune system mainly through immune cell receptors (TLR4). Activation of immune system causes increase in the levels of circulating cytokines and promotes development of inflammation and alcohol-mediated damage to internal organs [7].

Because activated immune cells generate ROS, the inflammatory process is always accompanied by oxidative stress [8, 9]. Impairments in the functioning of antioxidant and immune defense systems caused by the long-term alcohol consumption contribute to the formation of concomitant somatic pathologies in patients, which significantly reduces the efficacy of standard treatment. New therapeutic approaches have been developed based on the existing data on molecular mechanisms underlying the damaging effect of ethanol on the cells of body organs and put into practice. In particular, they include supplementing a standard therapy with drugs aimed at the molecular targets involved in the toxic effects of ethanol in order to increase the efficacy of used treatment [10]. An information on the fundamental processes involved in AD pathogenesis is essential for the improvement of already existing and development of new treatment methods. It can be obtained by studying oxidative stress at different stages of AD and identifying its clinical and biological associations.

The aim of this work was to investigate peripheral markers of oxidative stress in AD patients in the early abstinence period and to identify their relationship with AD clinical parameters and inflammatory factors.

MATERIALS AND METHODS

Eighty-four male patients with alcoholism admitted to the clinic of the Research Institute of Mental Health with a diagnosis coded by ICD-10 as “mental and behavioral disorders as a result of alcohol consumption (dependence syndrome F10.21 and withdrawal syndrome F10.30)” were studied. The patients were 30 to 60 years old (average age, of 44.3 ± 8.2 years), with the disease duration of 13.6 ± 8.0 years. Diagnostic assessment and clinical verification of diagnosis were performed by physicians of the Department of Addictive States. For each patient, a standardized description card was filled that contained general identifying data, clinical parameters, such as patient’s age at the time of examination, age of the alcohol withdrawal syndrome (AWS) formation, disease duration (calculated as a period of time from the AWS formation to the date of examination), and duration of AWS after alcohol withdrawal. As a control, we used blood samples of 80 conditionally healthy men (average age, 40.9 ± 9.6 years) who did not have problems with alcohol consumption, as well as chronic somatic diseases in the acute stage or symptoms of acute infectious diseases. Thirty-seven control blood samples were used to obtain plasma for the assessment of oxidative stress markers; the other 43 samples were used to obtain blood serum for the assessment of inflammatory mediators.

Blood from alcoholic patients was collected on days 3-5 of their admission to the hospital after alcohol detoxification performed to remove toxic substances from the body and to reduce the symptoms of AWS. Peripheral venous blood from AD patients and control individuals was collected in the morning on an empty stomach using a sterile one-use holder Vacutainer system (Becton Dickinson, USA) in heparin-coated tubes (for plasma) or tubes containing coagulation activator (for serum). The blood was centrifuged; serum and plasma were aliquoted and stored at –80°C until use.

The content of protein oxidation products in blood plasma was evaluated from the concentration of protein carbonyls (PCs; nmol/mg protein) using a method based on the reaction of PC derivatives with 2,4-dinitrophenylhydrazine (Panreac, Spain) with the formation of 2,4-dinitrophenylhydrazones [11]. Total protein concentration (mg/ml) was determined using a Total Protein 120 kit (Cormay, Poland). The content of LPO products were assessed from the concentration of thiobarbituric acid-reactive products (TBA-RPs; nmol/ml) using a TBA AGAT kit (Agat-Med, Russia). The content of the oxidative DNA damage product 8-hydroxy-2′-deoxyguanosine (8-OH-dG) was measured with a DNA Damage Competitive Elisa Kit (Thermo Fisher Scientific, USA) and expressed in ng/ml. The optical density of the oxidation products was determined with an EPOCH spectrophotometer (BioTek Instruments, USA).

Blood serum was analyzed for the concentration (pg/ml) of the following proinflammatory cytokines: interferon gamma (IFNγ); interleukin-1β (IL-1β); interleukin-6 (IL-6); interleukin-8 (IL-8); interleukin-17A (IL-17A); tumor necrosis factor alpha (TNFα). The assays were carried out with a MAGPIX multiplex analyzer (Luminex, USA) (Medical Genomics Center for Collective Use, Tomsk National Research Medical Center)

using MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel reagent kits (Merck, Germany).

Statistical analysis was performed with SPSS v. 23.0 for Windows; the data were presented as median and interquartile range Me [Q_L-Q_U], except for the age of study participants and disease duration that were presented as mean ± standard deviation (M ± SD). The data were examined for the normality of distribution using the Shapiro–Wilk test. The Mann–Whitney test was used for the between-group comparison. Correlation analysis was performed using the Spearman's rank correlation coefficient. Correlations and differences between the groups were considered significant at $p < 0.05$.

RESULTS

Table 1 shows the clinical data for the patients in the study.

The study was performed in alcoholic patients during the early abstinence period after alcohol detoxification.

Table 2 shows the results of comparative study of peripheral oxidative stress markers in patients with AD and healthy individuals.

Table 3 shows the concentration of proinflammatory cytokines (inflammatory mediators) in alcoholic patients.

Table 1. Clinical parameters of alcoholic patients (n = 84); Me [Q_L-Q_U]

Parameters	Value
Age, years	44.50 [38.00-50.00]
Age of AWS formation, years	30.00 [25.00-35.00]
Disease duration, years	12.50 [7.00-19.00]
AWS duration, days	4.00 [2.00-4.00]

The primary objective of our study was to identify the relationship between the oxidative stress markers, clinical parameters, and content of inflammatory cytokines in the blood of patients with AD. The results of correlation analysis are shown in Table 4.

DISCUSSION

Assessment of peripheral markers of oxidative stress (products of lipid, protein, and DNA oxidation in blood plasma) in patients with alcoholism in the early abstinence period revealed the development of a pronounced oxidative stress, as the concentrations of PCs and TBA-RCs in the plasma of alcoholic patients were significantly higher than in the control individuals (Table 2). A similar increase in the content

Table 2. Peripheral markers of oxidative stress in the plasma of alcoholic patients and healthy individuals; Me [Q_L-Q_U]

Oxidative stress marker	Alcoholic patients, n = 84	Control, n = 37	p between-group
PCs, nmol/mg protein	0.42 [0.35-0.49]	0.33 [0.27-0.39]	<0.001
TBA-RPs, nmol/ml	2.97 [2.50-3.55]	2.40 [2.10-2.80]	<0.001
8-OH-dG, ng/ml	13.30 [9.84-16.08]	12.84 [11.16-16.45]	0.908

Table 3. Concentrations of proinflammatory cytokines in the blood of alcoholic patients and healthy individuals; Me [Q_L-Q_U]

Inflammatory cytokine	Alcoholic patients, n = 31	Control, pg/ml; n = 43	p-value
IFNγ	32.46 [29.36-35.44]	3.07 [0.88-5.47]	$p < 0.001$
IL-1β	5.56 [4.91-6.41]	0.68 [0.25-2.46]	$p < 0.001$
IL-6	5.96 [4.74-10.47]	1.26 [0.35-4.06]	$p < 0.001$
IL-8	26.38 [19.72-40.53]	8.59 [5.69-13.78]	$p < 0.001$
IL-17A	24.27 [22.53-27.82]	2.83 [1.07-5.17]	$p < 0.001$
TNFα	30.03 [28.55-38.67]	9.75 [7.00-12.49]	$p < 0.001$

Table 4. Correlation of peripheral oxidative stress markers with clinical parameters and content of inflammatory cytokines in alcoholic patients

Parameters	Spearman's rank correlation coefficient (r_s)	*p*-value
TBA-RP and disease duration	0.201	0.049
PCs and age of AWS formation	–0.245	0.025
PCs and AWS duration	–0.246	0.024
PCs and IL-6	–0.620	0.003
8-OH-dG and IL-6	0.433	0.044
TBA-RPs and IL-8	0.457	0.049
TBA-RPs and TNFα	0.468	0.037

of peripheral oxidative stress markers, such as malondialdehyde (LPO product) and PCs in the serum of patients with alcoholism has been reported in the works of other authors [12, 13].

At the same time, the content of 8-OH-dG (product of DNA oxidation) in AD patients did not differ from the control (Table 2). Previously, we reported an increased concentration of 8-OH-dG in the blood plasma of AD patients with the AWS upon their admission to the hospital [14]. Alcoholic patients with the AWS or after 1 week of detoxification were also demonstrated an increased level of 8-OH-dG [15]. This discrepancy can be explained by the fact that the studies were conducted in patients with different stages of the disease, as well as by a variety of mechanisms that can affect the level of DNA oxidation products in human blood plasma. Chernikov et al. [16] analyzed the reports on the biomedical properties, mechanisms of action, and possible therapeutic effects of such DNA metabolites [16]. More than 30 oxidation products of nitrogenous bases are known, the most studied of which are 8-oxo-7,8-dihydroguanine (8-oxo-dG) and 8-OH-dG. Formation of 8-oxo-dG is the most common type of oxidative damage of nucleic acids [17], while 8-OH-dG is one of the main oxidative stress biomarkers [15, 18]. Free 8-oxo-dG has been shown to act as a mediator of stress signaling in cells that triggers and potentiates inflammatory and immune responses in order to maintain homeostasis upon the action of external agents. 8-OH-dG also exhibits pronounced anti-inflammatory and antioxidant properties [16]. Marmiy and Esipov in their review [19] on the biological role of 8-oxo-2′-dG and, in particular, its antioxidant and anti-inflammatory properties, cited a number of reports, in which the content of 8-oxo-2′-dG first increased under the action of stress factors, but then returned to the normal values or even decreased. The activity of antioxidant and DNA repair enzymes significantly increases during the compensation period. The question whether DNA oxidation products upregulate expression of such enzymes or activation of the corresponding genes is caused by some other factors remains open. However, beside the fact that 8-OH-dG has been successfully used as an oxidative stress marker for a long time, there is evidence suggesting involvement of this compound in the regulation of gene expression, DNA repair, control of inflammatory and autoimmune responses, and triggering of the antioxidant defense [19]. In view of this, the absence of differences in the levels of 8-OH-dG in the plasma of patients with AD and control subjects in our study is quite understandable. This metabolite not only accumulates in the body during oxidative stress, it is actively involved in the regulation of metabolic processes, which, in turn, can affect its concentration in blood plasma.

Assessment of proinflammatory cytokine levels showed that the concentrations of all studied inflammatory mediators in the blood serum of alcoholic patients were significantly higher than in the control individuals ($p < 0.001$ in all cases). The highest increase (by more than an order of magnitude) was found for the concentration of IFNγ (Table 3). Previously, the studies of spontaneous production of cytokines (IFNγ, IL-17A, IL-1Ra, IL-1β, IL-2, IL-6, IL-8, and TNFα) by immunocompetent cells revealed a significant increase in the concentration of cytokines in the supernatants of blood samples from patients with AD compared to healthy individuals [20]. There was a correlation between the spontaneous production of cytokines by blood cells of patients with AD and products of protein and lipid oxidation in the blood plasma of these patients [21], which indirectly indicated a stimulating effect of ethanol and its metabolic products on immunocompetent cells *in vivo*. At the same time, the products of ethanol-induced oxidative stress can modulate the synthesis of cytokines differently, as was demonstrated in the alcohol consumption model in rats by assessing the role of alcohol-induced oxidative stress in the modulation of cytokine production in hepatocytes and liver Kupffer cells [22]. The effect on the synthesis of proinflammatory cytokines/chemokines was mediated by the activity of NF-κB transcription factor, histone acetylation, and impaired mRNA stability. Thus, hydrogen peroxide (H_2O_2) stimulated LPS-induced cytokine/chemokine production in Kupffer cells, while 4-hydroxynonenal displayed the inhibitory effect.

Studying the correlations between oxidative stress markers and clinical characteristics of AD revealed negative associations of PC concentration with the

age of AWS formation and AWS duration after alcohol withdrawal (Table 4). Since mechanisms of AD formation and development involve multiple molecular pathways, it is impossible to explain the identified relationships unambiguously. Nevertheless, we can definitely say that PCs participate in these mechanisms.

We also found a direct relationship between the level of LPO products (TBA-RPs) and disease duration. It is known that aging is associated with the increased amounts of biomacromolecule oxidation products [23-25]. Spiteller [25] provided evidence that LPO are formed as a result of processes induced by changes in the structure of cell membrane that occur in aging, as well as under the influence of adverse factors, in particular, systematic long-term alcohol consumption. Structural changes in biological membranes under the action of ethanol lead to the activation of membrane-bound phospholipases, which induces the influx of Ca^{2+} ions and activation of lipoxygenases. Lipoxygenases convert polyunsaturated fatty acids into lipid hydroperoxides that can be degraded by enzymes into signaling compounds. This induces non-enzymatic LPO, and the process of TBA-RP accumulation continues [24]. We found a correlation between the content of TBA-RPs and disease duration in patients with AD.

The correlations between the oxidative stress markers and the content of proinflammatory cytokines found in our study confirm a close relationship between inflammation and oxidative stress [8, 9]. The relationship between the oxidative stress markers and IL-6 is of special significance, since IL-6 is the most important inflammatory mediator that induces the synthesis of acute phase proteins. We noted that the strongest associations among identified correlations were the inverse relationship between PCs and IL-6 and direct relationship between 8-OH-dG and IL-6 (Table 4). As already mentioned, 8-OH-dG has been found to exhibits pronounced antioxidant properties [16, 19]. One of the explanations for the observed associations may be the following: the inflammatory process upregulates IL-6, as well as promotes oxidation of plasma macromolecules, including DNA, with the formation of oxidation products (8-OH-dG), thus leading to the direct relationship between the levels of IL-6 and 8-OH-dG. Since 8-OH-dG has the antioxidant effect, its accumulation in the blood prevents protein oxidation and reduces the concentration of PCs in the blood, which leads to the negative correlation between the IL-6 content and PC concentration observed in our study.

Our explanation for the association of oxidative stress markers with AD clinical parameters and inflammatory mediators is still a hypothesis. Further research and more data are needed to identify the relationship between the metabolic pathways at different stages of AD development.

CONCLUSION

We found that the early abstinence period in AD patients is accompanied by a pronounced oxidative stress and accumulation of products of protein and lipid oxidation in the blood.

We also revealed the correlation between the levels of oxidative stress markers and disease duration, age of AWS onset, and AWS duration, as well as between the contents of PCs, TBA-RPs, 8-OH-dG, and proinflammatory cytokines, the concentrations of which were significantly increased in patients with AD.

It was demonstrated that the studied peripheral oxidative stress biomarkers reflect a combined contribution of oxidative stress and inflammation to the AD pathogenesis. The obtained data expand the knowledge about pathogenesis of alcoholism and might be helpful in the development of new treatment methods that will include therapeutic agents aimed at normalizing the oxidative status and reducing inflammation.

Contributions. V.D.P., T.P.V., and N.A.B. developed the concept and supervised the study; E.V.E. and A.S.B. conducted the experiments; V.D.P., T.P.V., and E.V.E. discussed the study results and prepared the manuscript; T.P.V. and N.A.B. edited the manuscript.

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Ethics declaration. All procedures performed in human subjects met the ethical standards of the National Research Ethics Committee and the Helsinki Declaration of 1964 and its subsequent revisions or comparable ethics standards. Voluntary informed consent was obtained from each involved participant. The study was approved by the Local Ethics Committee at the Mental Health Research Institute of Tomsk National Research Medical Center. The authors of this work declare that they have no conflicts of interest.

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