

THE RELEVANCE OF OXIDATIVE STRESS STATUS IN ONE WEEK AND ONE MONTH ALCOHOL ABSTINENT PATIENTS

VAŽNOST OKSIDATIVNOG STATUSA KOD PACIJENATA POSLE JEDNE NEDELJE I JEDNOG MESECA APSTINENCIJE OD ALKOHOLA

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Summary

Background: Although it is generally accepted that there is an increased oxidative stress status in alcoholics, the separate relevance of oxidative stress following alcohol withdrawal is still not understood to this date. There are reports stating that the increased oxidative stress status in alcoholics may persist independently of the constant presence of alcohol intake, while on the other side, it was demonstrated that the antioxidant defense mechanism could significantly increase after alcohol withdrawal.

Methods: In the present work, we were interested in studying the relevance of oxidative stress status in the alcohol withdrawal processes, by determining some oxidative stress markers (two antioxidant enzymes: superoxide dismutase – SOD and glutathione peroxidase – GPX and a lipid peroxidation marker – MDA) after one week and one month of abstinence, as compared to the baseline and a control group of subjects.

Results: Our data confirmed the increased oxidative stress status in alcoholic patients and, more importantly, we demonstrated here a significant decrease of the oxidative stress status one week and one month following the withdrawal, as showed by a significant increase in the specific activity of

Kratak sadržaj

Uvod: Iako je poznato da kod alkoholičara postoji stanje pojačanog oksidativnog stresa, do sada nije zasebno utvrđena važnost oksidativnog statusa u apstinenciji. Neki izveštaji govore da stanje povišenog oksidativnog stresa kod alkoholičara može trajati nezavisno od stalnog unosa alkohola, dok je s druge strane pokazano da mehanizam antioksidantne odbrane može značajno ojačati posle prestanka konzumiranja alkohola.

Metode: U ovom radu nas je zanimalo da proučimo važnost statusa oksidativnog stresa u procesima odvikavanja od alkohola, putem određivanja nekih od markera oksidativnog stresa (dva antioksidantna enzima: superoksid-dismutaze – SOD i glutation-peroksidaze – GPX, kao i markera lipidne peroksidacije – MDA) posle nedelju dana i posle mesec dana apstinencije i poređenja sa nivoima na početku studije i u kontrolnoj grupi ispitanika.

Rezultati: Naši podaci potvrđuju postojanje pojačanog oksidativnog stresa kod alkoholičara. Štaviše, pokazali smo ovde da posle nedelju i posle mesec dana apstinencije dolazi do značajnog smanjenja oksidativnog stresa, koje se ogleda u značajnom porastu specifične aktivnosti SOD ($p < 0,003$) kao i sniženju nivoa MDA ($p < 0,019$). Ipak, u slučaju tri markera

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List of abbreviations: ANOVA, analysis of variance; DSM-IV-TR, Diagnostic and Statistical Manual of Mental Disorders IV Text Revision; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; NAC, N-acetylcysteine; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOS, NO synthase; ROS, reactive oxygen species; SEM, standard error of the mean; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; WST-1, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt.

SOD ($p < 0.003$), as well as by a decrease in MDA levels ($p < 0.019$). Still, in the case of all three markers of oxidative stress status which we determined, the levels after one week or one month of abstinence were significantly altered when compared to controls.

Conclusions: This suggests that severe and prolonged deficiency in the oxidative stress marker levels needs longer than one month of abstinence to normalize.

Keywords: alcohol, abstinence, oxidative stress

Introduction

Alcohol is the most widely consumed drug worldwide and its levels of mortality and morbidity are increasing constantly, considering that alcohol abuse has been associated with a vast range of pathologic conditions (1–4).

Oxidative stress is the condition arising from the imbalance between toxic reactive oxygen species (ROS) and antioxidant systems (5), being also recognized as a very important mechanism in a variety of pathologies (6) including neuropsychiatric disorders, as our group has previously demonstrated (7–11).

Although it is now generally accepted that there is an increased oxidative stress status in alcoholics, mainly expressed through a reduction in the general antioxidant activity (a distinct feature of chronic alcohol exposure) (12) as well as by increased levels of lipid peroxidation markers such as malondialdehyde (MDA) in both cerebrospinal fluid or peripheral venous blood (13), the relevance of oxidative stress following alcohol withdrawal is still not understood to this date. There are reports stating that the increased oxidative stress status from alcoholics may persist independently of the constant presence of alcohol intake, perpetuating a prooxidative condition in the body even during abstinence (14–16). On the other side, it was demonstrated that the antioxidant defense mechanism (as represented for example by superoxide dismutase – SOD, the first antioxidant enzyme in the way of the free radicals) could significantly increase after alcohol withdrawal (12, 17). In fact, for the specific activity of SOD, there is a multitude of controversial studies, indicating an increase following alcohol withdrawal (as mentioned before), as well as a decrease (18, 19) or modifications, irrespective of alcohol ingestion or abstinence, or the kind of treatment administered (12, 15).

Thus, in the present report, we were interested in studying the relevance of oxidative stress status in the alcohol withdrawal processes, by determining some oxidative stress markers (two antioxidant enzymes – SOD and glutathione peroxidase and a lipid peroxidation maker – MDA) after one week and one month of abstinence, as compared to the baseline (oxidative stress status at the time of entry into the study) and a control group of subjects.

statusa oksidativnog stresa koje smo odredili, nivoi posle nedelju i posle mesec dana apstinencije bili su značajno izmenjeni u poređenju sa kontrolnom grupom.

Zaključak: Ovo ukazuje da je potrebno više od mesec dana apstinencije kako bi se normalizovala ozbiljna i produžena deficijencija u nivoima markera oksidativnog stresa.

Ključne reči: alkohol, apstinencija, oksidativni stres

Methods

The study included 33 patients aged between 26 and 79 years (average 45.2 ± 3.58 years), all of them males. They fulfilled the Diagnostic and Statistical Manual of Mental Disorders IV Text Revision (DSM-IV-TR) diagnostic criteria of alcohol dependence and were scheduled to be admitted to the alcohol detoxification ward (no. II B) for further alcohol dependence rehabilitation in the Psychiatry University Hospital, Iasi, Romania. Alcohol consumption was stopped abruptly at admission.

Alcoholic patients without illicit drug use, chronic systemic disease or severe mental disorders were selected. They were all treated with diazepam or lorazepam and also supplemented with vitamins from the B group (B1+B6). The detoxification ward was a restricted environment for one week to one month for the patients included in the study, who received identical meals provided by the hospital. However, only 19 from the initial 33 patients remained in the ward for one month, for the final blood collection, since most of them decided to drop out of the study (withdrawal symptoms ameliorated and discharge at patients' request or refusal to give another sample of blood).

The control group ($n=18$) included healthy age- and sex-matched subjects without known physical or psychiatric illnesses which were identified by clinical interview and routine laboratory tests. They also did not meet the diagnostic criteria of alcohol abuse or dependence in the past nor abusive alcohol consumption during the previous two months.

The study was conducted according to provisions of the Helsinki Declaration and the local ethics committee approved the study. All the patients or their families signed the consent for the participation in this study.

Biochemical estimations

Blood samples were collected at baseline ($n=33$), after one week ($n=33$) and after one month ($n=19$), in the morning, before breakfast, allowed to clot and centrifuged immediately. Serum was aliquoted into Eppendorf tubes and stored at -40°C until measurement.

The classical biological markers of chronic alcoholism such as gamma-glutamyltransferase, aspartate transaminase and alanine aminotransferase were determined, being significantly higher in alcoholic patients at baseline, when compared to controls. Additionally, all these markers decreased significantly after 1 week and 1 month of detoxification (data not shown).

Superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) substrate (a water soluble dye) and xanthine oxidase using an SOD Assay Kit (FLUKA, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide anions) after 20 minutes of reaction time at 37 °C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

The glutathione peroxidase (GPX) activity was measured using the GPX cellular activity assay kit CGP-1 (SIGMA). This kit uses an indirect method, based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is indicative of GPX activity.

Malondialdehyde levels were determined by thiobarbituric acid reactive substances (TBARS) assay. Two hundred μ L of serum was added and briefly mixed with 1 mL of trichloroacetic acid at 50%, 0.9 mL of TRIS-HCl (pH 7.4) and 1 mL of thiobarbituric acid 0.73%. After vortex mixing, samples were maintained at 100 °C for 20 minutes. Afterwards, samples were centrifuged at 3000 rpm for 10 min and supernatant read at 532 nm. The signal was read against an MDA standard curve, and the results were expressed as nmol/mL.

Data analysis

The levels of oxidative stress markers (SOD, GPX and MDA) were statistically analyzed by using one-way analysis of variance (ANOVA). All results are expressed as mean \pm the standard error of the mean (SEM). *Post hoc* analyses were performed using Tukey's honestly significant difference test in order to compare baseline, one week and one month groups. F values for which $p < 0.05$ were regarded as statistically significant.

Results

Regarding the specific activity of SOD, which is of course the first enzyme in the way of ROS, we report here a significant decrease at both baseline time ($F(1.49)=54$, $p < 0.0001$), as well as in the case of measurements that were taken after one week ($F(1.49)=11$, $p=0.001$) or one month ($F(1.35)=17$, $p < 0.0001$) after withdrawal, when compared to our set of control subjects (Figure 1).

Moreover, when we applied the *post hoc* analysis, we observed significant differences between the baseline and one week ($p < 0.0001$) or baseline vs. one month groups ($p=0.003$), as well as between one week and one month groups ($p=0.003$) (Figure 1).

Also, in the case of the other antioxidant enzyme we determined here, GPX, we observed a significant decrease at the baseline time ($F(1.49)=7$, $p=0.01$), as well as after 1 week ($F(1.49)=9$, $p=0.004$) or one month ($F(1.35)=17$, $p=0.01$) after withdrawal, as compared to the control group (Figure 2).

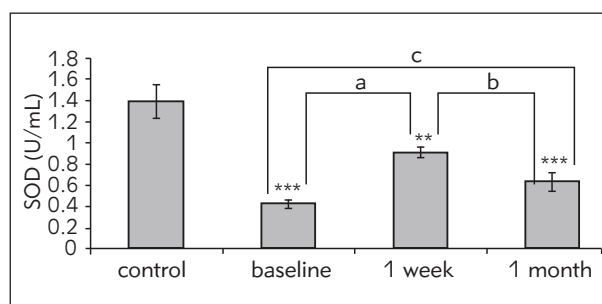


Figure 1 Superoxide dismutase (SOD) specific activity in the serum of control subjects, baseline and alcohol abstinent patients after one week and one month. The values are mean \pm SEM ($n=18$ in control group, $n=33$ in baseline and one week groups and $n=19$ in one month group). ** $p < 0.001$; *** $p < 0.0001$. For *post hoc* analysis – a (baseline vs. one week): $p < 0.0001$; b (one week vs. one month): $p=0.003$; c (baseline vs. one month): $p=0.003$.

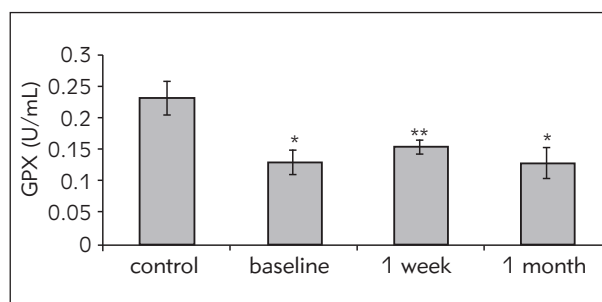


Figure 2 Glutathione peroxidase (GPX) specific activity in the serum of control subjects, baseline and alcohol abstinent patients after one week and one month. The values are mean \pm SEM ($n=18$ in control group, $n=33$ in baseline and one week groups and $n=19$ in one month group). * $p=0.01$; ** $p=0.004$.

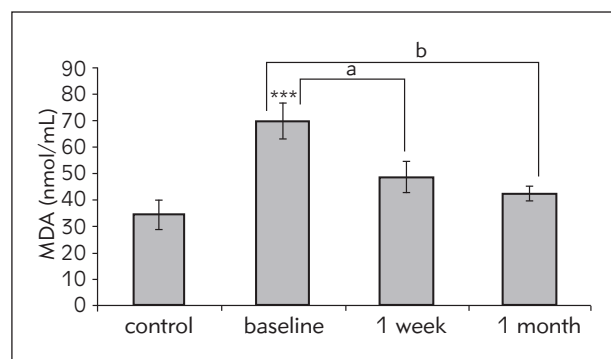


Figure 3 The levels of malondialdehyde (MDA) in the serum of control subjects, baseline and alcohol abstinent patients after one week and one month. The values are mean \pm SEM ($n=18$ in control group, $n=33$ in baseline and one week groups and $n=19$ in one month group). *** $p<0.0001$. For *post hoc* analysis – a (baseline vs. one week): $p=0.019$; b (baseline vs. one month): $p=0.004$.

Additionally, in this case, the *post hoc* analysis showed no significant differences between baseline and one week ($p=0.27$) or baseline vs. one month groups ($p=0.98$), as well as between one week and one month groups ($p=0.28$) (Figure 2).

When we determined the serum levels of MDA, as a fundamental parameter of the lipid peroxidation processes, we observed a very significant increase in the MDA levels at baseline ($F(1.49)=13$, $p<0.0001$), when compared to control subjects (Figure 3). Still, the levels of MDA were normal after one week ($F(1.49)=2$, $p=0.11$) and one month ($F(1.35)=1$, $p=0.2$), since no significant differences were observed in these two groups (one month and one week), when compared to controls (Figure 3).

Additionally, the *post hoc* analysis for MDA levels showed significant differences between baseline and one week determinations ($p=0.019$), as well as between baseline and one month ($p=0.004$). However, no significant differences regarding the concentrations of MDA were found between one week and one month determinations ($p=0.4$) (Figure 3).

Discussion

Our present data confirmed the increased oxidative stress status in alcoholic patients and, more importantly, we demonstrated here a significant decrease in the oxidative stress status one week and one month following the withdrawal, as showed by a significant increase in the specific activity of SOD, as well as by a decrease in MDA levels, when compared to baseline. As mentioned, we demonstrated an increased oxidative stress status in the patients with alcohol dependence, considering the reduced specific activity of both SOD and GPX, completed by the significant increase in MDA, as a specific marker of

the lipid peroxidation processes, when compared to our age-matched subjects.

These aspects are consistent with previous reports stating that the antioxidant activity is lower in chronic alcoholics than in control subjects. This was demonstrated for the antioxidant enzymes such as SOD, GPX and CAT (12, 19, 20), but also for the classic antioxidants such as glutathione (21). Also, the lipid peroxidation processes, as evaluated through the various levels of thiobarbituric acid reactive substances (TBARS), were reported to be significantly decreased in the CSF or blood of chronic alcohol consumers (16, 22, 23).

The mechanistic responsible for these effects are mainly represented by the mobilization of Fe^{3+} ions, acetaldehyde, as well as an alcohol-induced increase in NADPH-oxidases (1, 24, 25). Also, the alcohol-inducible cytochrome P450 isoform 2E1 (CYP2E1) seems to play a very important role in chronic alcohol consumers by generating an increased rate of ROS formation (19, 26, 27).

Regarding the withdrawal processes and relevance of oxidative stress in this matter, there are a lot of controversial results. In this way, as we previously mentioned for SOD, the first antioxidant enzyme against reactive oxygen species, both increased (12, 17) or decreased (16, 18) specific activity was reported following withdrawal.

Similarly, in the case of GPX, there are reports describing a significant decrease in its specific activity following withdrawal (12), while other authors failed to find any significant modification in GPX during abstinence (28). Also, for MDA, the main marker of the lipid peroxidation processes, while several studies have found elevated levels in the serum of patients undergoing alcohol withdrawal (20, 22, 29), there are also reports describing clear reductions in the various markers lipid peroxidation following alcohol withdrawal (12, 30).

Regarding the results we obtained in the present study, we report here a significant decrease in the oxidative stress status both one week and one month following the withdrawal, as showed by a significant increase in the specific activity of SOD, as well as by a decrease in MDA levels, when compared with those at baseline. Still, no significant modifications between the levels of GPX at baseline, one week and one month were observed. One possible explanation for this could be the fact that SOD is the most sensitive to and the primary defense against ROS damage (5), exhibiting prompt compensation processes when encountering or not heavy oxidative injury. Therefore, it is possible for the compensatory modifications of GPX to be less sensible, as compared to SOD.

However, in the case of all three markers of the oxidative stress status we determined here, the levels from one week or one month of abstinence were sig-

nificantly altered when compared to our age-matched group of control subjects, suggesting that severe and prolonged deficiency in their specific activity and levels needs longer than one month of abstinence to normalize. Therefore, additional experiments from our group are underway regarding the oxidative stress status in patients with alcohol dependence following 3, 6 or 12 months after withdrawal.

Also, despite some opinions that in fact the so-called detoxification of alcohol-dependent patients still leads eventually to a restoration of the prooxidant processes (16), it was also very recently demonstrated that oxidative stress could be relevant for the ameliorative processes that would actually appear after a period of abstinence in pathologies like alcoholic hepatitis (31). In this way, the Wang group showed that SOD, as well as glutathione levels, were increased in alcoholic hepatitis patients with abstinence, when compared to those without abstinence, while the levels of MDA were significantly decreased (31).

It seems that glutamatergic neurotransmission could be extremely relevant for the oxidative stress mechanisms during alcohol abstinence (18). Hence, Tsai et al. demonstrated that the augmentation of this excitatory neurotransmission may lead to enhanced oxidative stress, which together with reduced inhibitory neurotransmission may contribute to the symptoms of ethanol withdrawal and be associated with its neurotoxicity (18).

Another important aspect could be represented by the N-methyl-D-aspartate (NMDA) toxicity, which is known as an important mechanism in the alcohol withdrawal symptoms, by increasing both ROS and nitric oxide (NO), through the upregulation of NO synthase (NOS) (22, 32). This could be very important, considering that lately there is an increased awareness regarding the interactions that might exist between the oxidative and nitrosative stress status in various cellular insults (33). Moreover, as it was demonstrated in some animal studies, NOS inhibition resulted in an attenuation of alcohol withdrawal symptoms (19, 34).

Of course, all the aforementioned aspects lead to the idea of using antioxidants in order to ameliorate damage done by alcohol consumption and abstinence. Thus, N-acetylcysteine (NAC) was proposed for the improving of myocardial oxidative stress in alcoholic heart disease, an important pathologic condition associated with alcoholism. The protective effects of NAC could be also explained by the fact that it is required for glutathione biosynthesis, thus inhibiting ROS toxicity (35). Additionally, it was demonstrated that alcohol-induced oxidative stress could be

inhibited by NAC administration (36, 37). Moreover, it was showed that in fact NAC intake and ethanol abstinence interact synergistically in order to improve the hepatic antioxidant defenses and to decrease the serum lipids concentrations (37). It was also demonstrated relatively recently (38) that procysteine could increase alcohol-depleted glutathione stores in the plantaris muscle of some rat models, after a period of abstinence, considering that chronic alcohol abuse may lead to a variety of skeletal muscle complications, including atrophy, altered gait and impaired mobility (38).

There are, of course, several limitations of our present study, including the lack of vigorous control for the diet, body mass index and alcohol concentrations consumed before withdrawal, as well as the limited number of patients used and also the relatively short period of time chosen for the observation. Still, further studies regarding the aforementioned markers and their relevance in the present study are underway right now by our group, as well as additional experiments regarding the oxidative stress status in patients with alcohol dependence following 3, 6 or 12 months after withdrawal.

Another aspect could be represented by the fact that the patients took antioxidants such as the B group vitamins, but this could not be avoided since it represents a classical way to treat this kind of patients. Still, all the patients took without any exceptions exactly the same combination of B1+B6 vitamins.

Conclusions

Our present study confirmed the increased oxidative stress status in alcoholic patients (as showed by decreased antioxidant enzymes and increased lipid peroxidation). We also demonstrated here a significant decrease in the oxidative stress status one week and one month following the withdrawal, as showed by a significant increase in the specific activity of SOD, as well as by a decrease in MDA levels, when compared to baseline. Still, in the case of all three markers of the oxidative stress status which we determined (SOD, GX and MDA), the levels from one week or one month of abstinence were significantly altered when compared to controls, suggesting that severe and prolonged deficiency in their levels needs longer than one month of abstinence to normalize.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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