Progress in Neuro-Psychopharmacology & Biological Psychiatry 33 (2009) 66–69

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnpbp

The correlation between early alcohol withdrawal severity and oxidative stress in patients with alcohol dependence

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ARTICLE INFO

Article history:
Received 9 June 2008
Received in revised form 9 October 2008
Accepted 9 October 2008
Available online 27 October 2008

Keywords:
Alcohol withdrawal symptoms
Lipid peroxidation
Malondialdehyde
Oxidative stress
Superoxide dismutase

ABSTRACT

Oxidative stress is enhanced in alcoholic patients. This clinical study aimed to explore the correlation between alcohol withdrawal severity and two oxidative stress markers, malondialdehyde (MDA) and superoxide dismutase (SOD). Seventy-six inpatients fulfilled the DSM-IV-TR criteria for alcohol dependence and 19 healthy controls were enrolled. Serum MDA level and SOD activity were measured within 24 h of alcohol detoxification. The severity of alcohol withdrawal was evaluated by the Chinese version of the revised Clinical Institute Withdrawal Assessment for Alcohol Scale (CIWA-Ar-C) every 8 h. Average and highest scores of the CIWA-Ar-C at the first day were recorded as the baseline withdrawal severity. We compared the differences of MDA and SOD between groups, and examined the correlation between baseline withdrawal severity and oxidative stress markers. Compared to controls, serum MDA levels were significantly elevated and SOD activity was significantly lowered in alcoholic patients. In stepwise multiple regression analysis, MDA was the only variable significantly correlated with the average (β=0.48, p<0.0001) and highest (β=0.47, p<0.0001) CIWA-Ar-C scores at the first day of detoxification. In agreement with previous studies, alcoholic patients encountered high oxidative stress. Although there was a correlation between early withdrawal severity and MDA levels, the meanings of the correlation are worth further studies in the future.

1. Introduction

Alcohol is metabolized primarily by alcohol dehydrogenase and aldehyde dehydrogenase. In addition, the alcohol-inducible cytochrome P450 isoform 2E1 (CYP2E1), which is also expressed in the central nervous system, plays an important role in chronic alcoholics (Lieber and DeCarli, 1970). The high rate of CYP2E1 oxidative activity causes the formation of reactive oxygen species (ROS) and ethanol-derived (hydroxyethyl) free radicals, and can thus initiate lipid peroxidation (Cederbaum, 1989; Shaw, 1989). Lipid peroxidation reflects the interaction between oxygen molecules and polyunsaturated fatty acids, producing oxidation of various breakdown products of the latter. Among them, malondialdehyde (MDA) is a reliable marker of oxidative damage (Esterbauer et al., 1991; Shaw, 1989; Song, 1996). The chief antioxidant defense mechanism equipped in cells, superoxide dismutase (SOD), prevents superoxide from generating further toxic oxidative radicals resulting from alcohol consumption (Davis et al., 1990) and thus participates in the neutralization of ROS.

The excitatory neurotransmitters are involved in the adaptive up-regulation of *N*-methyl-D-aspartate (NMDA) receptors after chronic alcohol exposure (Bleich et al., 2004). NMDA-induced excitotoxic damage is mediated by its high Ca^{2+} permeability, evoked generation of ROS and subsequent enhancement of oxidative stress. Previous clinical studies have demonstrated that chronic ethanol consumption is accompanied by excessive oxidative damage and reduced levels of endogenous antioxidants (Lecomte et al., 1994; Peng et al., 2005). In addition, both excitatory neurotransmission and lipid peroxidation have been found to be significantly elevated in alcoholic patients (Bleich et al., 2000). Since oxidative stress and glutamate overactivation are regarded as sequential as well as interacting processes (Coyle and Puttfarcken, 1993), both of them have been postulated to contribute to neurobiological manifestations of chronic alcoholism (Crews et al., 2004).

Central excitotoxicity caused by overstimulation of NMDA receptors following alcohol cessation has long been implicated in

Abbreviations: ALT, alanine-aminotransferase; AST, aspartate-aminotransferase; AWS, alcohol withdrawal symptoms; CIWA-Ar-C, Chinese version of the revised Clinical Institute Withdrawal Assessment for Alcohol Scale; CYP2E1, cytochrome P450 isoform 2E1; D-Bil, direct bilirubin; GGT, gamma-glutamyltransferase; MDA, malondialdehyde; NMDA, *N*-methyl-D-aspartate; PTT, prothrombin time; ROS, reactive oxygen species; SOD, superoxide dismutase; T-Bil, total bilirubin.

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doi:10.1016/j.pnpbp.2008.10.009

the mechanism underlying alcohol withdrawal symptoms (AWS) (Lovinger, 1993; Nagy et al., 2001; Tsai et al., 1998). Importantly, increased glutamatergic neurotransmission after alcohol withdrawal has been shown to have a positive correlation with lipid hydroperoxide levels in human cerebrospinal fluid (CSF) (Tsai et al., 1998). This implies that AWS, a manifestation of central glutamate overexcitation, are linked with underlying oxidative damage. Thus, we hypothesized that AWS could clinically reflect the excessive oxidative stress. By measuring both serum MDA levels and SOD activity, we first examined alcoholic patients suffering from higher oxidative stress than control subjects in this study. Then we aimed to investigate the correlation between clinical withdrawal severity and MDA levels as well as SOD activity in alcoholic patients.

2. Materials and methods

2.1. Study subjects

This study was approved by the Institutional Review Board of Taipei City Psychiatric Center (TCPC). Patients who fulfilled DSM-IV-TR diagnostic criteria of alcohol dependence and who were scheduled to be admitted to the alcohol detoxification ward for further alcohol dependence rehabilitation programs in the TCPC routinely received a complete medical examination, including biological screening, by medical doctors. Those with known severe physical illnesses or those becoming ill before admission to the detoxification unit, such as severe liver cirrhosis or infection, were referred to other medical wards first for medical management. All of the alcoholic patients without illicit drug use, chronic systemic disease, or any clinical evidence of the abovementioned illnesses were invited to participate in the study. The control group included healthy 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 alcohol consumption during the previous three months. Written informed consent was obtained from all subjects after explaining the study goal and procedures.

2.2. Clinical assessment

The alcohol and tobacco use history was gathered for the alcoholic patients, including duration of alcohol dependence and average daily amount of alcohol consumption in the past one month. For all the recruited patients, alcohol consumption was stopped abruptly and completely at admission and their AWS were evaluated using the Chinese version of the revised Clinical Institute Withdrawal Assessment for Alcohol Scale (CIWA-Ar-C), which has been proved to be a reliable measurement for alcohol withdrawal symptoms (Huang et al., 2005), every 8 h by trained nurses. Average and highest scores of CIWA-Ar-C at the first day were recorded as the baseline withdrawal severity. Since alcohol withdrawal symptoms varied among individuals, in order to control the dose effect of drugs on CIWA-Ar-C scoring, we applied a standardized treatment protocol, i.e. a fixed-dose schedule of alcohol detoxification, by giving lorazepam 2 mg every 6 h for 4 doses in the first 24 h and then adjusting the lorazepam dose according to the CIWA-Ar-C flexibly thereafter. We also gave as-needed trazodone for sleep problems and vitamin B_1 150 mg/day. All patients received the same meals supplied by the hospital during admission.

2.3. Laboratory assays

Venous blood samples were obtained the next morning (am 8:00–9:00) of admission after an overnight fasting for measurements of MDA, activity of SOD, albumin, direct bilirubin (D-Bil), total bilirubin (T-Bil), gamma-glutamyltransferase (GGT), aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), and prothrombin time (PTT). The blood specimens were centrifuged and serum samples were stored at −80 °C until analysis.

2.3.1. Determination of MDA levels

The procedure was partly adapted from methods developed by Jentzsch et al. (1996). First, 0.5 ml of serum from the alcoholic patients or controls was mixed with 25 μl butylated hydroxytoluene (BHT; 88 mg/ml absolute alcohol) in ethanol. Then 2.5 μl 0.05 M sulfuric acid was added and the mixture was vortexed for 10 s, then left to stand for 10 min at room temperature. Next, 25 μl 0.2% thiobarbituric acid (TBA) was added and the mixture was vortexed again. The reaction mixture was then incubated at 100 °C for 30 min in a water bath. The tubes were then put on ice to stop the reaction. After standing at room temperature, thiobarbituric acid reactive substances (TBARS) were extracted with 3 ml of n-butanol. The n-butanol phase was centrifuged at 10,000 ×*g* for 5 min. The absorbance of the n-butanol extract phase was measured with a UV/VIS Spectrophotometer (Model V-550, Jasco International Co., Ltd., Tokyo, Japan) at 450 nm to 610 nm.

2.3.2. Determination of SOD activity

Cu, Zn-SOD levels were measured by an enzyme-linked immunoassay of the sandwich type using commercial kits and standard protocols (superoxide dismutase ELISA kit; ALEXIS Corporation Lausen, Switzerland) (Franke and Porstmann, 1994). Ten μl of serum from the alcoholic patients or controls and 50 μl of anti-SOD-HRP conjugate and 100 μl phosphate buffer saline (PBS, pH 7.4) were simultaneously added to the wells of a microtitration plate, which was coated with anti-SOD antibodies (5 μg/ml). The plate was then shaken for 15 min at room temperature. After washing the wells three times with washing buffer (phosphate buffered saline with 1% Tween 20, pH 7.4), tetramethyl-benzidine (TMB) substrate (Sigma, St. Louis, USA; 100 μl/well) was added and then the plate was incubated for 10 min at room temperature. The reaction was stopped by adding 100 μl of stop solution (1 M phosphoric acid) per well. Absorbance values were read at 450 nm in an ELISA reader (Model MRX; Dynex Technologies, Inc., Chantilly, VA, USA). For standard curve generation, a Cu, Zn-SOD standard was prepared. Serial fold dilutions (0.08, 0.16, 0.32, 0.63, 1.25, 2.0, 2.5 ng/ml) of Cu, Zn-SOD standard yielded a good correlation of

Table 1
Demographic, clinical characteristics and laboratory data in healthy controls and patients with alcohol dependence

	Controls		Patients with AD	
	n = 19		*n* = 76	
	Mean	SD	Mean	SD
Age*	30.4	10.4	41.2	8.5
Height (cm)	–	–	166.7[a]	5.6
Weight (kg)	–	–	62.7	14.2
Body mass index (kg/m^2)	–	–	22.5	4.8
Smoking (cig/day)	–	–	22.1[b]	13.0
Alcohol consumption (g/day)	–	–	208.9	100.7
Duration of alcohol dependence (years)	–	–	12.4[c]	7.7
GGT** (U/L)	24.2	7.3	434.8	558.9
AST** (U/L)	16.8	2.6	130.6	98.7
ALT** (U/L)	14.6	5.3	60.8	65.2
D-Bil (mg/dl)	–	–	0.3	0.4
T-Bil (mg/dl)	0.6	0.2	1.1[d]	0.7
Albumin (g/dl)	4.5	0.7	4.3	0.4
PTT (s)	–	–	29.1	3.4
MDA** (μM)	4.0	0.9	9.2	5.5
SOD** (ng/ml)	159.6	105.3	28.3	23.3

Abbreviations: AD, alcohol dependence; GGT, gamma-glutamyltransferase; AST, aspartate-aminotransferase; ALT, alanine-aminotransferase; D-Bil, direct bilirubin; MDA, malondialdehyde; PTT, prothrombin time; SOD, superoxide dismutase; T-Bil, total bilirubin.
[a,b,c,d] are respectively based on 75, 70, 72, 73 available data.
* $p<0.05$ by *t*-test.
** $p<0.05$ by analysis of covariance, age as a covariance.

SOD activity (SOD activity = 0.965X + 0.027, where X = dilution factor of the Cu, Zn-SOD standard; r^2 = 0.9966; data not shown).

2.4. Statistical analyses

Data were expressed as the mean and standard deviation (SD). We used a t-test or analysis of covariance (ANCOVA) to test the differences between various numerical variables between the alcoholic and control groups. Logarithmic transformation was used to make the distribution of biological indexes become normal. Stepwise multiple regression analysis was used to estimate the linear relationship between the severity of AWS and various clinical variables and biochemistry indexes. The severity of AWS, expressed as average and highest CIWA-Ar-C score at the first day of detoxification was treated as a dependent variable, and various clinical variables and biological indexes as independent variables. A p value of less than 0.05 was considered to have statistical significance. Analyses were carried out using statistical software (SPSS 12.0).

3. Results

Seventy-six patients and 19 healthy control subjects were recruited. Table 1 shows the demographic and clinical characteristics, and laboratory data of both groups. The alcoholic group was older than the healthy control group. It was apparent that the classical biological markers of chronic alcoholism were significantly elevated in the alcoholic patients. The MDA level in the alcoholic group was significantly higher than that in the control group, while SOD activity was significantly lower.

The average as well as highest CIWA-Ar-C scores (SD) at the first day of detoxification were 7.8 (5.3) and 11.7 (7.8) which significantly positively correlated with serum MDA level (regression coefficient β = 0.48 and 0.47 respectively, $p < 0.0001$), but not with SOD, GGT, AST, ALT, albumin, D-Bil, and T-Bil levels or PTT, nor other clinical variables, including age, body mass index, duration of alcohol dependence, mean of daily alcohol consumption and smoking amount. Fig. 1 demonstrates the correlation between the highest first-day CIWA-Ar-C score and serum MDA level after logarithmic transformation.

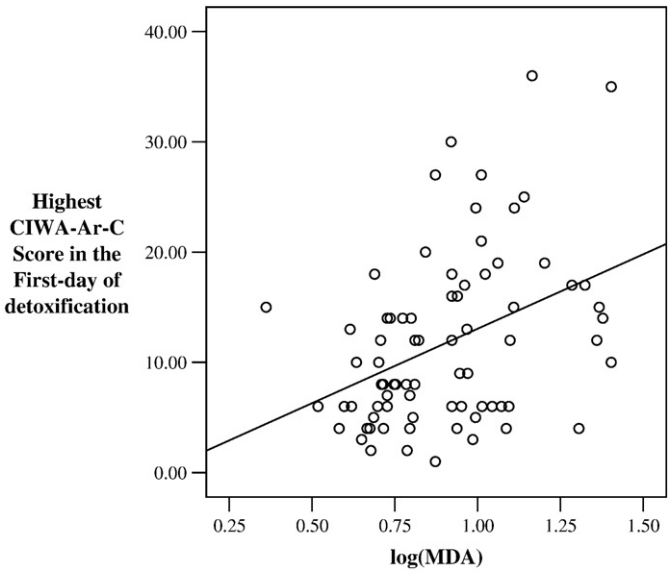

Fig. 1. The correlation between highest first-day alcohol withdrawal severity evaluated by the Chinese version of the revised Clinical Institute Withdrawal Assessment for Alcohol Scale and malondialdehyde levels after logarithmic transformation in alcoholic patients (n = 76) (regression coefficient: β = 0.47, $p < 0.0001$). Abbreviations: CIWA-Ar-C, Chinese version of the revised Clinical Institute Withdrawal Assessment for Alcohol Scale; log(MDA), malondialdehyde levels after logarithmic transformation.

4. Discussion

Our data showed a significant elevation of MDA levels and reduction in SOD activity in alcoholic patients compared to healthy controls. Notably, the clinical withdrawal severity (average or highest scoring of first-day CIWA-Ar-C) was significantly positively correlated with serum MDA levels after controlling other variables, with modest correlation coefficient. To our knowledge, this is the first paper addressing the relation between clinical withdrawal symptoms and oxidative stress.

The increase in lipid peroxidation and defective antioxidant mechanism in the alcoholic patients was in agreement with previous reports. Using lipid hydroperoxides in the cerebral spinal fluid (CSF), Tsai et al. (1998) observed persistently higher than normal lipid peroxide levels and reduction in SOD activity after alcohol withdrawal. From the analysis of peripheral blood, several studies also consistently found elevated MDA levels as well as weakened SOD activity in patients with chronic alcoholism undergoing alcohol withdrawal (Bleich et al., 2000; Lecomte et al., 1994; Marotta et al., 1997; Peng et al., 2005; Soardo et al., 2005; Yuksel et al., 2005). This implies that patients with chronic alcohol dependence have difficulty in compensating for alcohol-induced excessive production of free radicals resulting in toxicity and organ damage, especially in the central nervous system (CNS). Moreover, due to the characteristics of rich polyunsaturated fatty acids, poor SOD and catalase activity (Halliwell and Gutteridge, 1990), the brain is highly susceptible to alcohol-induced oxyradical injury, which may partly mediate various neurobiological symptoms.

The study demonstrated that clinical withdrawal severity was significantly correlated with the extent of heavy lipid peroxidation. It is difficult to understand the exact association of central oxidative stress and withdrawal phenomenon. Elevated generation of ROS by alcohol may disrupt the phospholipids of cell membranes, leading to or enhancing successive pathological changes. NMDA excitotoxicity, characterized in the mechanism for AWS, is considered to interact with oxidative stress to further potentiate cell damage and neurotoxicity (Lipton and Rosenberg, 1994). Meanwhile, NMDA activation increases not only ROS but also nitric oxide (NO) by upregulating NO synthase (NOS) (Gunasekar et al., 1995). In animal studies, NOS inhibition, which decreased NO formation, could attenuate the AWS (Adams et al., 1995; Uzbay et al., 1997). Therefore, the role of NO in the expression of AWS has been suggested (Uzbay and Oglesby, 2001). One clinical study exhibited elevation of serum NO and MDA levels during alcohol withdrawal (Yuksel et al., 2005), but the correlation between serum NO level and AWS severity is still unclear. Taken together, oxidative stress may participate in the presentation of AWS. Supporting this hypothesis, it has been found that augmented glutamatergic neurotransmission correlated positively with, or may lead to, oxidative damage (Tsai et al., 1998) during alcohol withdrawal. In addition, elevation of both serum homocysteine, a representation of excitatory neurotransmission, and MDA levels have also been noted in alcoholic patients (Bleich et al., 2000). In parallel, the present study, by measuring AWS directly, provides clinical evidence that withdrawal symptoms, a manifestation of underlying glutamatergic overexcitation, may be a possible reflection of excessive lipid peroxidation.

The study has some limitations. First, despite the correlation between AWS severity and MDA levels being significant after we had controlled the effects of clinical parameters and other biochemical variables, including liver function, the correlation coefficient (0.48) was modest in stepwise multiple regression analysis. This warrants future studies to verify the findings and suggests that lipid peroxidation is potentially affected by some physical problems subclinically, such as mitochondrial alterations (Hirano et al., 1992), early liver damage after chronic alcohol use, or coexisting hepatitis infection. Although no significant difference was found between oxidative indexes between hepatitis B carriers and non-carriers in our alcoholic

subjects (data not shown), we still cannot eliminate the effects from other organic factors on MDA levels. Second, we did not measure diet, alcohol concentrations, and genetic polymorphism of alcohol-metabolizing genes for patients. Therefore, the possibility of these factors affecting the current findings cannot be ruled out. Third, the control group was apparently younger than the alcoholic group. However, we used statistical analysis (ANCOVA) to control the age effect between groups and the differences of MDA levels and SOD activity between the control and alcoholic groups still remained significant. We suggest that the possibility of an age effect on the differential expression of oxidative stress between the groups might be minimal. Fourth, we did not have the data for body mass index in the control group and thus could not evaluate the impact of obesity on oxidative stress among these subjects. However, in the alcoholic group, no significant correlation was found between oxidative indicators and body mass index (data not shown).

5. Conclusions

Alcoholic patients suffer from excessive oxidative stress. Their clinical withdrawal severity during early withdrawal was correlated with the extent of lipid peroxidation. The biological meanings of the correlation remain to be studied in the future.

Acknowledgements

This work was supported by grants from the National Science Council (NSC94-2314-B-532-001, NSC94-2314-B-038-067, and NSC95-2314-B-532-008) and the Department of Health, Executive Yuan (DOH94-TD-M-113-048 and DOH95-TD-M-113-041), Taiwan.

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