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Altered oxidative stress/antioxidant status in blood of alcoholic subjects is associated with alcoholic liver disease

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ABSTRACT

Background: Oxidative stress is implicated in pathogenesis of alcoholic liver disease (ALD). This study investigated the possible correlation among the erythrocyte indices of oxidative stress, the leukocyte panels of antioxidant proteins (metallothioneins), the serum biochemical parameters and the liver steatosis grade.
Methods: A total of 118 cases including 60 alcoholic subjects and 58 controls were enrolled. All the alcoholic subjects were screened for body mass index (BMI), liver steatosis, and blood chemistry and serology. The level of oxidative stress and oxidative stress-related parameters were measured in the blood and correlated with clinical findings.
Results: Alcoholic subjects showed higher BMI, moderate/severe hepatic steatosis, increase in the levels of triglycerides, cholesterol, glucose, γ-glutamyl-transpeptidase (GGT), alanine aminotransferase (ALT), bilirubin, alpha 1 and beta 2 globulins, iron and a decrease in the levels of aspartate aminotransferase (AST) and beta 1 globulin with respect to the reference values. Moreover, alcoholic subjects showed: (i) an increase in Thiobarbituric Acid Reactive Substance (TBARS) content representing a good estimation of global oxidative stress; (ii) a stimulation of the activities of the antioxidant enzymes catalase and SOD; (iii) a modulation of expression of metallothioneins, with a down-regulation of MT-1A and an up-regulation of MT-1E isoforms.
Conclusions: Our data suggest that alcoholism is strongly associated with altered pattern of blood metallothioneins; this parameter combined with the score calculated by an *ad hoc* implemented algorithm (HePaTest) could offer a non-invasive alternative approach for evaluating alcohol-related damages and could be used in follow-up of alcoholic patients.

1. Introduction

Alcohol-related diseases affect millions of individuals and are an important cause of morbidity and mortality worldwide. Alcoholic liver disease (ALD) is major cause of liver transplantation and death; it encompasses a large spectrum of conditions ranging from simple steatosis through steatohepatitis up to cirrhosis (Orman et al., 2013; Testino, 2013). Although the majority of heavy drinkers develops steatosis, only in a minority of patients there is a progression of liver damage, depending on additional risk factors such as gender, genetic, metabolic and nutritional factors (Seth et al., 2011).

Liver biopsy is the gold standard to assess the severity of hepatosteatosis, but its invasiveness may lead to possible complications (Dhanda et al., 2013). Recent studies have demonstrated that hepatic ultrasonography (US) is highly sensitive and specific to estimate the degree of steatosis, thus representing an alternative to biopsy (Hernaez et al., 2011). Hematological analyses are widely used for evaluation of ALD severity: some parameters are associated with liver functionality (e.g., albumin and bilirubin), some with hepatocyte integrity (e.g., transaminase), and some with conditions linked to oxidative stress (gamma-glutamyl transferase; Chalmers et al., 1981; Pratt and Kaplan, 2000; van Beek et al., 2014).

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Oxidative stress is typically associated to fatty liver, and it is a feature of the ethanol hepatoxicity that contributes to the progression of liver damage also through its association with inflammation and immune reactions (Albano, 2002; Shinde et al., 2012; Vidali et al., 2008). Oxidative stress results from an imbalance between production and destruction of Reactive Oxygen Species (ROS; Sies, 1991). ROS are reactive molecules that attack the most important biopolymers such as lipids, proteins and DNA (Halliwell, 1999). Excess ROS induce lipid peroxidation reactions that trigger degenerative processes affecting cell membranes (Albano, 2006; Ishii et al., 1997; Wu and Cederbaum, 2009); lipid peroxidation typically increases in ALD patients. Administration of antioxidants or reduction of unsaturated fatty acid consumption prevents lipid peroxidation thus reducing inflammation in alcohol-fed rats (Nanji, 2004).

Antioxidant defenses act in protecting cells from the different species of oxidants. ROS are scavenged by enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx; Mates et al., 1999), as well as by non-enzymatic antioxidants such as glutathione (GSH), metallothioneins (MTs) and vitamins. In particular, MTs are highly-conserved (Vergani, 2009), low-molecular weight (6–7 kDa), cysteine-rich proteins with high affinity for divalent metals (Kagi, 1991; Vasak and Meloni, 2011). MTs mainly act in homeostasis of essential and detoxification of toxic metals, but also in scavenging of free radicals (Sato and Bremner, 1993; Vergani et al., 2005). MTs seem to modulate complex diseases in humans. In rats assuming high fat diet, fatty liver shows increased levels of MTs and increased oxidative stress, thus pointing at a direct correlation between MT induction and oxidative status in the hepatic cell (Grasselli et al., 2008). Recently, our group reported that binge ethanol administration to rats led to a marked induction of hepatic MT expression (Grasselli et al., 2014). Moreover, MT-overexpressing transgenic mice are significantly protected from hepatic oxidative stress associated with alcoholic liver injury (Zhou et al., 2002). The involvement of MTs in the protection against alcohol-induced oxidative damage is further supported by data showing that zinc supplementation prevents alcoholic liver injury in mice through MT induction and oxidative stress attenuation (Zhou et al., 2005). Although the importance of oxidative stress in ALD is widely accepted, many steps in the pathways leading to liver damage need to be elucidated.

Here, we measured the level of oxidative stress and oxidative stress-related parameters in the blood of 118 subjects including 60 alcoholic patients and 58 controls. Peripheral blood is considered a good reporter for the oxidative status of the whole organism (Albano, 2006). All the alcoholic subjects were screened for body mass index (BMI), liver steatosis, and blood chemistry and serology. Moreover, we implemented and tested a novel algorithm (HePaTest) that is comprehensive of the serum parameters strictly related to liver status. HePaTest is negatively related with MT-1A expression, and positively related with serum Iron level.

2. Materials and methods

2.1. Subjects

A total of 118 adults aged 18–60 years were enrolled and divided into two groups. The alcoholic group (AL) included 60 subjects, 15 women (mean age: 54 ± 13 years) and 45 men (mean age: 48 ± 10 years) who referred to the specialized Centro Alcologico Regionale, (CAR) of San Martino Hospital in Genoa. Control/social drinker group (C) included 58 apparently healthy volunteers, 18 women (mean age: 52 ± 17 years) and 40 men (mean age: 46 ± 14 years). All patients and healthy controls were Caucasian, except one patient who was Ecuadorian. Inclusion criteria included a well-documented history of ethanol consumption either continuously (more than 10 units/day for at least 20 years) or during repeated episodes of binge drinking (1–14 units/week for women, and 1–21/week for men). Documentation of ethanol intake was based on personal interviews using a time-line follow-back technique. All patients abstained to drink alcohol and the mean duration of abstinence prior to sampling was 2 ± 2 days. Alcoholic subjects met DSM-IV criteria for current alcohol dependence as assessed by the Structured Clinical Interview (First et al., 2014). Patients were screened for blood chemistry and serology and BMI (kg/m^2). The patients were in pharmacology therapy for alcoholism consisting of metadoxine (300 mg), vitamins (2500 mg cyanocobalamin, 0.70 mg folic acid and 12 mg nicotinamide) and antioxidants (646 mg glutathione) administered intravenously for 1 month (5 days/week), followed by a cycle of metadoxine and glutathione (3 days/week for 2 weeks) and metadoxine and glutathione (2 days/week for 2 weeks). Finally, therapy was suspended in case of abstinence. In three selected cases, therapy with anti-craving (disulfiram 400 mg/day for 6 months) and psychoactive (Diazepam 10 drops/day for 1 month) drugs as domiciliary care was also administered.

Alcoholic subjects who had a history of chronic systemic disease, inflammatory disease, HAV, HBV or HCV infections or severe liver injury were excluded. Healthy controls were recruited from the same community, and reported no lifetime or current history of any drug dependence (including alcohol) and no psychiatric disorders. The blood samples from alcoholic subjects were collected during the periodic clinical check. Blood samplings in the control group, all volunteers, were carried out during periodic medical examination. All procedures involving human subjects were approved by the Local Committee and are in accordance with the Helsinki declaration of 1975.

2.2. Liver ultrasonography

To assess grading and staging of liver steatosis, hepatic ultrasonography (US) is the most common modality (Dasarathy et al., 2009). Hepatic US was performed by a single radiologist ultrasonographer using an ultrasound system (EsaoteMyLab 70 XVG, Esaote, Genoa, Italy) with linear and convex transducers (frequency bandwidth 2.5–3.5 MHz). The ultrasonographer was blinded to the clinical, laboratory and histological data of the patients. Ultrasonographic steatosis score (USS) was calculated according to Hamaguchi et al. (2007).

2.3. Blood sample collection

Blood was collected into vacutainer tubes containing anticoagulants; all manipulations were carried out within 2 h after sample collection. Leukocyte (WBC), erythrocyte (RBC) and plasma fractions were separated by centrifugation over Ficoll-Hypaque (Histopaque®-1077). Then, WBCs were collected and resuspended in Trizol for RNA extraction. RBCs were washed twice with physiologic solution and stored at −80 °C.

2.4. Determination of lipid peroxidation

Lipid peroxidation in RBC homogenates was determined through the thiobarbituric acid reactive substances (TBARS) spectrophotometric assay based on the reaction of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA) (Kawamoto et al., 2005). Briefly, 2 vol. of TBA solution (0.375% TBA; 15% trichloroacetic acid; 0.25 N HCl) were added to 1 vol. of the RBC lysate and incubated for 45 min at 95 °C. Then, 1 vol. of N-butanol was added and the organic phase was read using a UV–vis Varian Cary50 spectrophotometer (Agilent, Milan, Italy). The MDA level was expressed as nmol MDA/mg Hb (Grasselli et al., 2008).

2.5. Determination of antioxidant enzyme activities

RBCs were lysed in ice-cold deionized water (Vergani et al., 2011). Hemoglobin (Hb) content, measured according to Drabkin (1949), was used to normalize the enzyme activities. In hemolysates diluted in 50 mM phosphate buffered saline (PBS) pH 7.0, catalase activity was evaluated at 25 °C following the decomposition of H_2O_2 and expressed as micromoles of decomposed H_2O_2 per min/mg Hb (Aebi, 1984). In aliquots of hemolysate treated to eliminate the Hb interference, SOD activity was measured as the inhibition of the reduction rate of cytochrome c by the superoxide radical and expressed as mU/mg Hb (McCord and Fridovich, 1969). Data are the mean ± SD of three experiments in duplicate.

2.6. RNA isolation and real-time RT-PCR

Total RNA was isolated using the Trizol reagent (Vergani et al., 2011); cDNA was synthesized as previously described (Grasselli et al., 2012). The expression levels of MT genes were quantified in 96-well Chromo4™ System PCR apparatus using iTaq-SYBR Green Supermix (Biorad, Milan, Italy). Using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene, the relative gene expression was calculated following the comparative Ct threshold method (Pfaffl, 2001). Data are expressed as fold induction with respect to the controls. Data are the mean ± SD of three experiments in quadruplicate.

2.7. Statistical analysis

Data were tested for normal distribution (D'Agostino–Pearson test) and, unless stated otherwise, presented as medians with interquartile range (25th and 75th percentiles). Data are visualized as Box and Whisker Plots. Average values from alcoholic subjects were compared with those from controls using Mann–Whitney