Blood thiamine, zinc, selenium, lead and oxidative stress in a population of male and female alcoholics: clinical evidence and gender differences

Rosanna Mancinelli(a), Eleonora Barlocch(i)(a), Maria Ciprotti(b), Oreste Senofonte(c), Rosanna Maria Fidente(a), Rosa Draisci(a), Maria Luisa Attilia(d), Mario Vitali(d), Marco Fiore(e) and Mauro Ceccanti(d)

(a) Centro Nazionale Sostanze Chimiche; (b) Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare; (c) Dipartimento di Ambiente e Connessa Prevenzione Primaria, Istituto Superiore di Sanità, Rome, Italy; (d) Centro di Riferimento Alcologico Regione Lazio, Sapienza Università di Roma, Rome, Italy; (e) Istituto di Biologia Cellulare e Neurobiologia, Consiglio Nazionale delle Ricerche, Rome, Italy

Abstract

Introduction. Long term alcohol abuse is associated with deficiencies in essential nutrients and minerals that can cause a variety of medical consequences including accumulation of toxic metals. Aim. The aim of this research is to get evidence-based data to evaluate alcohol damage and to optimize treatment. Thiamine and thiamine diphosphate (T/TDP), zinc (Zn), selenium (Se), lead (Pb) and oxidative stress in terms of reactive oxygen metabolites (ROMs) were examined in blood samples from 58 alcohol dependent patients (17 females and 41 males).

Results. T/TDP concentration in alcoholics resulted significantly lower than controls (p < 0.005) for both sexes. Serum Zn and Se did not significantly differ from reference values. Levels of blood Pb in alcoholics resulted significantly higher (p < 0.0001) than Italian reference values and were higher in females than in males. ROMs concentration was significantly higher than healthy population only in female abusers (p = 0.005).

Conclusion. Alcoholics show a significant increase in blood oxidative stress and Pb and decrease in thiamine. Impairment occurs mainly in female abusers confirming a gender specific vulnerability.

INTRODUCTION

Alcohol abuse may cause physical, mental and social injuries and is considered a serious public health concern in Italy where epidemiological data show increasing consumption and at-risk drinking behaviors. This justifies the great alarm about alcohol problems which have a heavy impact on the present and future public health [1]. Alcohol abuse can produce systemic alterations in the body, including an unbalance of essential elements such as thiamine (vit B1), zinc (Zn) and selenium (Se) due to impairment of the homeostatic mechanisms [2]. This happens as a consequence of malnutrition, lowered absorption and a decreased intestinal transport. Alcohol abuse may reduce availability of thiamine (T) which plays an essential role in brain function and alcohol related thiamine deficiency is strongly involved in alcohol related brain damage. Furthermore T is involved in the elimination of some toxic metals, as in the case of Pb, where T is essential for metal elimination and for limiting the damage caused by it [3]. Previous studies demonstrated deficiency of thiamine and thiamine diphosphate (T and TDP) in alcoholic patients mostly in females, and the results were significant enough to candidate determination of thiamine as a marker of alcohol abuse [4, 5]. Zn is found in almost every cell of the human body. It is a component in about 200 enzymes, and it is therefore involved in more enzymatic reactions than any other mineral. Furthermore, Zn plays a critical role in the immune system where it helps to regulate the production and activity of T-lymphocytes and natural killer cells. Selenium is required in very small amounts and it is a powerful antioxidant. It is part of

Key words
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• oxidative stress

Address for correspondence: Mancinelli Rosanna, Centro Nazionale Sostanze Chimiche, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail:rosanna.mancinelli@iss.it.
the glutathione peroxidase enzyme that works closely with the antioxidant vitamins C and E to neutralize free radicals, i.e. the unstable, highly reactive molecules that may cause damage to human cells. Metabolic impairment due to alcohol abuse may affect the levels of these essential elements and also the metabolism of some toxic compounds [6-8], so that the exposure to environmental substances considered safe for the general population may be unsafe for heavy drinkers. In this clinical study it has been examined also the blood concentration of Pb since lead is one of the most toxic metals for human health and one of the most widespread in the environment [9]. Furthermore previous studies associated high blood Pb levels with alcohol drinking [10-15]. The hypothesis is that alcohol drinking may contribute to Pb accumulation also reducing availability and/or activity of essential elements. There are different routes of exposure to Pb from contaminated air, water, soil, food, and consumer products. Pb is a ubiquitous and potent neurotoxicant, induces several neurophysiological and behavioural changes and, even if alters the function of multiple organs and systems, it primarily affects the central nervous system. In adult humans, encephalopathy resulting from Pb intoxication is often characterized by sleeplessness, poor attention span, vomiting, convulsions and coma; in children, Pb-induced encephalopathy is associated with mental dullness, vomiting, irritability and anorexia; diminished cognitive function resulting in a mental deficit has been also observed during lead intoxication. Prolonged exposure to Pb can produce oxidative stress, disrupt the blood-brain barrier and alter several Ca++-dependent processes, including physiological processes that involve nitric oxide synthesis [16-18]. Intriguingly, many of the symptoms due to Pb intoxication look like alcohol-related effects, so it’s difficult to distinguish if they are due to ethanol or to Pb accumulation and misdiagnosis may therefore hinder the correct treatment. We intend to explore by this study if the two situations could be concomitant and strictly related.

Metabolic impairment due to alcohol abuse promotes a further negative effect: the generation of the so-called "reactive oxygen species" (ROS) interfering with the physiological processes that take place mainly in the liver [19-21]. The involvement of free radical mechanisms in alcohol-induced liver pathogenesis has been shown by the detection of oxidative markers in the liver and serum of alcoholics, but the mechanisms of alcohol toxicity are still not completely understood. Reactive oxygen species (ROS) are naturally generated in small amounts during metabolic processes affecting liver functions. No females could be pregnant or exposed to contraceptive drugs.

Reference values for T/TDP were obtained from 45 males and 58 females healthy social drinkers randomly drawn from subjects attending the “Prevention and Work Security Service” for routine medical examination. Their mean age was 42.94 ± 10.17 years (men 45.8 ± 11.4), the mean lifetime length of alcohol abuse was 20.8 ± 13.2 years with a significant gender difference: females 14.0 ± 12.0 years, males 23.2 ± 12.8 years (p = 0.05). Patients were divided into three groups on the basis of the prevalent type of alcoholic beverage accounting for more than 50% of total alcohol intake: i) wine drinkers, ii) beer drinkers, iii) spirit drinkers, to verify a possible effect due to the type of alcohol drink. Wine drinkers were 60% of the patients and abuse had lasted 23.6 ± 14 years; beer drinkers were 18.2% and abuse had lasted 15.5 ± 8.9 years; spirit drinkers were 12.7% and abuse had lasted 19.9 ± 13.7 years. About 9% of patients declared no drinking preference.
by the Istituto Superiore di Sanità in the years 2008-2010 in cooperation with the Italian Blood Donors Volunteer Association (AVIS). PROBE is the population study carried out to determine metals’ exposure of the healthy population in Italy. The internal dose of 20 metals including Pb was examined in a sample consisting of about 1400 Italian adults, aged 18-65 years and living in five different Italian regions. The mean values were Pb = 24.0 µg/l with gender differences: males = 26.2 µg/l; females = 19.6 µg/l [23].

For the evaluation of oxidative stress in terms of reactive oxygen metabolites (ROMs), the reference values of the d-Roms test was established by the manufacturer on a sample of about 5000 healthy subjects. The reference range was 250-300 U CARR corresponding to 20.0-24.0 mg H2O2/dl. When blood values are > 300 U CARR the subject is considered to be under oxidative stress.

All blood samples were taken early in the morning after overnight fasting. For the determination of T/TDP and Pb requiring whole blood, the samples were collected in tube containing EDTA as anticoagulant. For the determination of Zn, Se and ROMs that requires serum, the samples were collected in tubes without anticoagulant. Blood samples were accompanied by a personal datasheet containing socio-demographic and alcohol consume information.

**Analytical procedures**

**Thiamine**

T/TDP were assessed in the erythrocytes by high-performance liquid chromatography (HPLC) procedure with pre-column alkaline oxidation to thiochrome and final fluorimetric detection under isocratic condition.

Blood samples (7 ml) were collected in EDTA vacutainers tube and processed afterwards. After plasma separation, the red blood cells were washed three times with four volumes of saline in graduated tubes: cells were pelleted at 1000 g for 10 min at 10 °C and the buffy coat removed to carefully eliminate TDP-rich leukocytes. Finally, cell suspension was centrifuged at 1400 g for 20 min at 10 °C to obtain well packed red blood cells and then the saline was partially removed leaving a 1:1 cell suspension, thus obtaining all the analytical samples with final hematocrit 48-50%. Extraction: the cell suspension was added with trichloroacetic acid 40% and the obtained extract was subjected to derivatization with potassium hexacyanoferrate. Sample was thereby processed by HPLC with fluorescence detection operated at 375 nm excitation and 430 nm emission. Detailed information about the analytical procedure are reported in a previous paper [4].

**Zinc and selenium**

Determination of Zn in the serum was performed by flame atomic absorption spectroscopy (F-AAS) after dilution of the samples. Standard solutions for calibration were prepared using 1:5 glycerol to simulate the viscosity of the samples.

Selenium in the serum was assessed by inductively coupled plasma mass spectrometry (ICP-MS). Serum samples were diluted 1:10 using (v/v) high-purity deionized water and analyzed without digestion procedure. Selenium determination was performed by collision/reaction cell Elan DRC II (Perkin Elmer SCIEX, Norwalk, CT, USA). Selenium determination in serum by DRC-ICP-MS requires previous selection of the isotope masses virtually free of polyatomic interferences. In the case of selenium, Ar dimers at m/z 78 (40Ar-38Ar+) and 80 (40Ar2+) overlapped the selenium masses; 82Se is usually interference-free, but unfortunately it is only 8.73% abundant, so 78Se (23.78%) was preferred. In order to reduce isotopic interference, the instrument was operated in reaction mode using H2 gas to eliminate Ar dimers. In this mode, selenium reacts very slowly with H2, but the Ar2⁺ interference is rapidly moved to one mass higher (Ar3⁺) and away from 78Se.

**Oxidative stress**

Oxidative stress was evaluated in serum by colorimetric determination of reactive oxygen metabolites (ROMs) by means of a specific d-Roms Test and a dedicated instrumentation (DIACRON INTERNATIONAL Srl). In the d-Roms Test, reactive oxygen metabolites in the biological samples produce radicals R-O• and in turn R-OO• in the presence of iron released from plasmatic proteins by an acid buffer. These radicals react with the kit reagent imparting a pink derivative quantified by a photometer. Results are expressed as U CARR, where 1 U CARR = 0.08 mg H2O2/dl. This test was chosen among the available ones because of its diagnostic sensitivity and specificity and because the analytical procedure is easy to perform to be introduced in the routine laboratory tests [24]. The test is based on the concept that the amount of organic hydroperoxides present in the serum is related to the free radicals from which they are formed. When the serum sample is dissolved in an acidic buffer, the hydroperoxides react with the transition metal ions liberated from the proteins in the acidic medium and are converted to alkoxo and peroxy radicals. These newly formed radicals are able to oxidize an additive (N,N-diethyl-para-phenylenediamine) to the corresponding radical cation. The concentration of this persistent species can be easily determined through spectrophotometric procedures (absorption at 505 nm). The reference values of the test are between 250 and 300 U CARR (Carratelli Units), where 1 U CARR corresponds to 0.8 mg H2O2/dl.

**Lead**

Determination of Pb in the whole blood was performed using Zeeman graphite furnace atomic absorption spectrometry (Z-GFAAS) after acid digestion of the samples. The blood sample was weighed directly into vials that had previously been decontaminated. After adding 1 ml H2O2 and 6 ml concentrated HNO3, the sample was evaporated to 0.3 ml on a thermo-regulated hot-plate by gradually increasing the temperature to 150 °C. After cooling, the solution was diluted to a volume of 3 ml by adding high-purity water (resistivity value < 18 MΩ/cm). All glassware and polyethylene containers were pre-rinsed using 5% v/v nitric acid and high-purity water to eliminate surface contamination. The solutions of treated samples were stored in polypropylene vials until analysis. Certified reference material ClinChek-Control-WholeBlood was used as control.
Values outside this range are considered indicative of an alteration in the equilibrium between pro-oxidant and antioxidant capability of subjects. Values > 300 U CARR indicate a condition of oxidative stress.

**Statistical analysis**

Statistical evaluation involved the t-test between means, the one-sample t-test and the regression analysis. Statistical significance: p < 0.05.

**RESULTS**

The comparison of data of alcoholics vs controls are described in Figure 1. The mean of T was not different between males and females alcoholics, 63.0 nmol/l ± 27.3 and 62.7 nmol/l ± 28.7 respectively, but it was significantly lower than the controls = 89.6 ± 22.7 nmol/l (p < 0.001). The mean of TDP was 161.1 ± 97.3 nmol/l in male and 142.9 ± 48.3 nmol/l in female alcoholics; these values were significantly lower than that found in controls = mean 222.23 ± 56.3 nmol/l (p < 0.005 by paired sample t-test).

The mean of Zn serum levels in alcoholics was 856 ± 171 µg/l, and felt into reference range (600-1080 µg/l). Only two alcoholic patients presented values below the reference range (341 and 297µg/l) were found in 12 patients (20.6%). The one-sample t-test showed no significant differences for Zn and Se. The Pb concentration in alcoholics resulted 69.8 ± 41.0 µg/l and a major content of Pb was found in females (76.9 ± 47.0 µg/l) than in males (67.3 ± 38.5 µg/l). In 16 men and 8 women, i.e. the 41.3% of the studied population, blood Pb was over than 80 µg/l that is more than threefold the mean of Pb in Italian general population (24.0 µg/l). The statistical analysis performed by one sample t-test showed significant differences for both gender: p < 0.001. When the type of alcohol drink was considered, it was found that blood Pb in wine drinkers (81.5 ± 38.5 µg/l) was higher than in spirit drinkers (39.1 ± 22.3 µg/l, p < 0.008) and beer drinkers (46.6 ± 22.1 µg/l, p < 0.01). Spirit and beer drinkers were not significantly different. Furthermore, mean blood Pb was increasing with the years of alcohol abuse: Pb = 47.7µg/l (3-10 years of abuse); Pb = 69.3 µg/l (11-20 years of abuse); Pb = 82.5 µg/l (21-30 years of abuse) and Pb = 94.1 µg/l (30-49 years).

Mean oxidative stress in terms of ROMs resulted 285.55 ± 75.66 U CARR and was significantly different from the reference range 250-300 U CARR (one-sample t-test: p = 0.05). In 7 alcoholic patients ROMs values were in the range 341-400 U CARR (slight oxidative stress) and 3 patients in the range 401-500 U CARR (severe oxidative stress). Noticeably, when data were analyzed separately for sex, oxidative stress in female alcoholics resulted significantly higher (p < 0.005), while in males was not: p = 0.17 (Figure 2). The 12 patients with lowest selenium concentration showed concomitant condition of high oxidative stress (mean = 384.7 ± 65 U CARR). The multivariate analysis showed correlation of oxidative stress with Pb and Se(**) where correlation was significant at the 0.01 level (**Table 1**). The multiple regression analysis performed by setting blood Pb as the dependent variable, resulted p = 0.05 for Pb vs. age, and p = 0.002 for Pb vs oxidative stress. (Table 2). In a nutshell, gender comparison shows damage in alcoholic women which does not occur equally in men.

**DISCUSSION**

Research on the ethanol metabolism established that alcohol is hepatotoxic, mainly because of secondary malnutrition and ethanol oxidation. These effects are due to redox changes produced by nicotinamide adenine dinucleotide (NADH) generated via the liver’s alcohol dehydrogenase (ADH) pathway which in turn affects the metabolism of lipids, carbohydrates, proteins and purines. In addition to ADH, ethanol can be oxidized by
liver microsomes by the ethanol-inducible cytochrome P450 (CYP2E1) which contributes to ethanol metabolism and tolerance and to the selective hepatic perivenular toxicity of various xenobiotics. This may explain the increased susceptibility of heavy drinkers to the exposure to industrial solvents, anesthetic agents, commonly prescribed drugs, chemical carcinogens and even nutritional ingredients such as vitamin A. The induction of the microsomal pathway in case of alcohol abuse contributes to increase acetaldehyde generation and significant impairment in the ability of the liver to utilize oxygen thus enhancing negative effects due to

**Figure 1**
Comparison between controls and alcoholics

*The figure shows the differences between controls and male and female alcoholics for all the studied parameters. Significant differences are marked by the symbol.*

**Figure 2**
Gender differences in oxidative stress and Pb content

*The figure shows that female oxidative stress and Pb levels are higher than in males. Significant differences are marked by the symbol.*
concomitant professional or environmental exposure to other toxins. Today the dyad lifestyle-environment plays a more significant worldwide role for health than ever before but, in toxicological studies related to work environments, alcohol drinking is still considered a risk factor only for its acute effect on performance (pilots, car drivers, bus drivers, industrial workers). There are very few data about health damage due to professional and/or environmental toxic exposure in heavy drinkers and toxicological studies rarely consider ethanol intake [25]. When this happens, alcohol intake is usually evaluated by self-report data even though the reliability of this method is still a matter for debate [26]. Alcohol drinking should be detected by specific clinical examinations and biomarkers such as blood alcohol concentration (BAC), transaminases (aspartate transaminase (AST) and alanine transaminase (ALT)), mean corpuscular volume (MCV), gamma-glutamyl-transferase (GGT) and carbohydrate deficient transferrin (CDT). Unfortunately, even though traditional and new biomarkers have been identified for different targets of alcohol related problems, there is not yet a “gold standard” for the diagnosis of alcohol abuse, and different combinations of two or more markers have been proposed to enhance the diagnostic power [27]. Some alcohol biomarkers are modified by concomitant exposure to other toxins and may not be able to fully discriminate between the different effects of alcohol [28]. Furthermore dangerous lifestyle patterns such as smoking, drug abuse and alcoholism may act together with environmental toxins thereby promoting neurotoxicity, alteration to the gamma-aminobutyric acid (GABA) system, mitochondrial damage, impairment of the immune system and teratogenic effects [29-32].

The results of the present study confirm that essential nutrients as T/TDP are reduced in alcoholics. TDP is reduced above all in female patients and this condition may significantly contribute to alcohol related risk for women health. In fact, during pregnancy thiamine deficiency due to alcohol intake may become heavier for hyperemesis gravidarum, i.e. the excessive vomiting during pregnancy that causes severe dehydration, weight loss, fluid and electrolyte imbalance for the mother and could affect fetus development. It's well known that there is a great variability of alcohol effects on fetus; this may be explained by a lot of reasons including genetics and environment but deficiency of essential nutrients as thiamine can concur to fetus impairment even by a small intake of alcohol. The teratogenic mechanisms of alcohol aren't yet completely understood so, up to today, a “safe” drinking in pregnancy doesn’t exist and the unique recommendation is to stop drinking at all. Zn and Se levels were not significantly different from reference values, whilst blood Pb in alcoholics resulted significantly higher than general population. Pb is the second on the list of dangerous substances indicated by the Agency for Toxic Substances and Disease Registry (ATSDR) in 1999 and the International Agency for Research on Cancer (IARC) [33], and many regulatory limits have been set for lead in food, water, soil, etc. even though new sources of lead contamination are continuously created by the environment [34-36]. The toxicity of Pb is well known since ancient times, but recently, like many other pollutants the dose considered dangerous has been significantly decreased. In low doses Pb is rapidly excreted, but when the dose is high a part remains immobilized in bone and hair where it is considered to be relatively harmless. In higher doses, Pb is accumulated in the liver causing harmful effects including neurological effects (encephalopathy, cognitive damage, antisocial behaviour), loss of appetite and teratogenic effects as Pb passes through the placenta during pregnancy and reaches the fetus where it may damage the developing central nervous system [37, 38]. In 2011 the reference values of Pb in Italy were quantified in blood samples from about 1400 male and female blood donors (age range 18-64 years) collected during the year 2008-2010 [18]. The values found in this last study are lower than that obtained ten years ago and this is presumably due to the reduced Pb content in the gasoline [39]. Women, besides of their lowest content of T/TDP, resulted more vulnerable to Pb accumulation and this evidence shows a further risk factor since deficiency of thiamine may contribute to negative effects of alcohol including Pb accumulation in the blood. In fact thiamine is considered an effective antidote against lead intoxication for its ability to bind and remove Pb from the tissues. Recently published data obtained by spectroscopic studies demonstrated that Pb interacts with the pyrimidine ring of thiamine leading to its solubilization at physiological pH [40]. The pyrimidine ring of thiamine mediates its interaction with Pb, prevents Pb accumulation and increases clearance from the tissues. In the present study, probably for the small size of the sample, statistical analysis did not show correlation between Pb and T/TDP and further studies in this field are needed. Results show that blood Pb increases with the number of years of alcohol abuse, suggesting an accumulation effect, and that in wine drinkers Pb was higher than in spirit and beer drinkers: this seems to indicate wine as an “independent” risk factor. The importance of the type of alcoholic drink is consistent with previous results by animal experimentation about prenatal alcohol exposure [41]. Two groups of mice pups, prenatally exposed to red wine and aqueous solution at the same ethanol concentration, were compared. Results showed that ethanol solution but not red wine induced behavioral and brain neurotrophin alterations in young and adult mice. This suggests that substances other than ethanol may contribute to alcohol impairment and that the pattern of alcohol drinking may significantly modulate alcohol effects. Finally some consideration about oxidative stress. As expected, oxidative stress was high in many patients according to the well-known capability of ethanol to trigger overproduction of ROS and to impair antioxidants defense [42, 43]. Intriguingly, in our study when data were examined separately for gender, it resulted that women were significantly affected by oxidative stress (p = 0.005) whilst men not (p = 0.17) notwithstanding the length of female alcohol abuse was significantly shorter than males. This evidence confirms a specific gender vulnerability.
CONCLUSION

Globally, the results of this clinical study highlight that toxic effects of alcohol are various and contribute in different way, including Pb accumulation, to impair homeostasis. Impairment is stronger in women and seems to be related not only to alcohol intake but also to the type of alcoholic drink. Further studies are required to understand why this happens. Surely, there is yet a lack of knowledge about alcohol damage and data suggest that concomitant exposure to alcohol and environmental substances may enhance negative effects of alcohol itself. It’s likely that future clinical studies about alcohol problems will have to consider this aspect and new research will be carried out in the field of “environmental diseases” as it’s happening, for example, for the research on cancer. In the light of the evidence-based data obtained by this study, to support findings of this study.

REFERENCES


32. Flora SJ, Kumar D, Sachan SR, Das Gupta S. Combined exposure to lead and ethanol on tissue concentration of essential metals and some biochemical indices in rat liver microsomes and Golgi apparatus. Toxicology 1991;81(2):157-64. DOI: 10.1007/BF02863081


