Abstract. Background: The alterations of total nitric oxide (NO) (through total nitrite/nitrate) and inducible nitric oxide synthase (iNOS) concentrations were determined in a population of alcohol-dependent individuals without liver disease upon admission for detoxification, two weeks later and after completion of detoxification (4-6 weeks in total).

Materials and Methods: Thirty-eight men and nine women were included in the study. Endogenous nitrite and total nitrite/nitrate concentrations were measured colorimetrically and iNOS concentration was measured by enzyme-linked immunosorbent assay (ELISA).

Results: Endogenous and total nitrite concentrations were found to be diagnostically equally conclusive, whereas iNOS values were not correlated with the other two parameters. All three parameters were significantly higher in alcohol-dependent individuals compared to controls at all time points. Conclusion: The preventive therapeutic use of iNOS inhibitors in alcohol-dependent individuals might avoid the injurious effects of chronic alcohol abuse, and should be a matter of further investigation.

Nitric oxide (NO) is an important intra- and intercellular mediator. Its radical was first identified as the endothelial-derived relaxing factor, the principal signal for vascular smooth muscle cell relaxation (1). In addition, NO regulates diverse biological activities, such as neurotransmission, tumor cell killing, immunity and inflammatory processes (2, 3).

NO is produced via the reaction of L-arginine, NADPH and oxygen, catalyzed by the enzyme oxide synthase (NOS), producing NO and citrulline (4). In mammals, there are three distinct genes encoding an equal number of NOS isozymes or isoforms: neuronal (nNOS or NOS1) found in neuronal cells, endothelial (eNOS or NOS3) found in endothelial cells and the inducible (iNOS or NOS2) found in macrophages, chondrocytes and hepatocytes (5). iNOS and nNOS are soluble molecules found predominantly in the cytosole, while eNOS is membrane-associated.

The iNOS isoform is induced by cytokines and endotoxin, producing large amounts of NO as a defense mechanism mainly against parasites, bacterial infection and tumor growth. It is also involved in the pathogenesis of septic shock, as well as in autoimmune diseases and ectopic pregnancy (5-7). Altered concentrations of NO have been found to be associated with inflammatory processes related to sepsis, reproduction, infection, hypertension, exercise, type 2 diabetes, hypoxia, and cancer (8-10).

In chronic alcohol abuse, alcoholic liver disease (ALD) is caused by endotoxemia associated with intestinal barrier leakiness and increased intestinal permeability, through disruption of tight junctional proteins and damage of the microtubuli, a result of oxidative injury based on ethanol stimulation of the iNOS. However, the exact mechanism of alcohol-induced iNOS-mediated disruption is unknown (11-13). It has been shown that a redox-sensitive transcription factor (SNAIL), stimulating epithelial mesenchymal transition (EMT), is activated by iNOS in gut leakiness triggered by alcohol use. EMT is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells. It is characterized by increased expression of mesenchymal cell markers such as vimentin, as well as by decreased expression of epithelial cell markers.
such as the cell–cell junctional proteins E-cadherin, occludin and the claudins (14–17).

NO is a gaseous free radical with a short in vivo half-life of a few seconds, which is converted to the very stable metabolites nitrite and nitrate. Thus, plasma nitrite and nitrate concentrations are used as markers for activity of NOS and the total production of NO radicals. In laboratory practice, plasma nitrite determination is meaningless because nitrite is rapidly oxidized to nitrate. Thus, first endogenous plasma nitrite is measured, then nitrate is enzymatically converted to nitrite, and the total nitrite concentration is measured (18).

The aim of the present study was to investigate the alterations of total NO (through total nitrite/nitrate) concentration and iNOS activity in a population of alcohol-dependent individuals without liver disease upon admission to an inpatient alcohol detoxification program, two weeks later, and after the completion of detoxification (4-6 weeks in total), as well as to assess their correlations at these time points.

**Patients and Methods**

Forty-seven (n=47) alcohol-dependent individuals (38 men and nine women) were included in the study. Participants were enrolled over a one-year period and had consecutively contacted the Drug and Alcohol Addiction Clinic of the Eginion University Hospital in Athens, Greece. All patients fulfilled the Diagnostic and Statistical Manual of Mental Disorders DSM-IV-TR diagnostic criteria for alcohol abuse/dependence and were admitted to this specialized unit for inpatient alcohol detoxification (19). Patients had abstained from alcohol for a mean±SD of 24.0±12.2h prior to their admission. Written informed consent was obtained from participants; permission for the study was obtained from the Ethics Scientific Committee of the Eginion Hospital, and the procedures followed were in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983. Inclusion criteria were: (i) 22-76 years of age; (ii) absence of serious physical illnesses, as assessed through physical examination and routine laboratory screening; (iii) absence of another pre- or coexisting DSM-IV-TR axis I major mental disorder; however, if symptoms of disturbed mood were present concurrently with alcohol abuse, patients were not excluded from the study, and (iv) absence of other substance abuse, with one exception: nicotine.

The participants were diagnosed by the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) and assessed through the Composite International Diagnostic Interview (CIDI) for their pattern of alcohol abuse, potential major life problems related to alcohol consumption and the occurrence of withdrawal symptoms in the past (20-23). Sociodemographic and psychiatric history data were also recorded. Details on patient assessment are provided in a previous study published by our group (24). A control group consisting of 160 healthy blood donors (138 men and 22 women) was used for comparisons.

Alcohol detoxification lasted approximately for a week (range=7-10 days). Vitamin supplementation (B complex, C, E) and oral diazepam (30-60 mg daily in divided doses), with gradual tapering off over 1-2 weeks, were given, followed by a four- to five-week inpatient treatment program with short-term psychotherapy of cognitive-behavioral orientation.

Fasting blood was obtained within 24 h upon admission for detoxification, two weeks later and after completion of the detoxification protocol (4-6 weeks). Hepatic enzymes serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyvic transaminase (SGPT), Gamma-glutamyl transferase (γGT), glucose, Thyroid-stimulating hormone TSH and mean corpuscular volume, or "mean cell volume" (MCV) were measured using conventional automated laboratory diagnostic methods.

Endogenous nitrite and total nitrite/nitrate concentrations were measured by means of a commercial Total Nitric Oxide kit (R & D Systems, Minneapolis, USA, Supplied by Anti-cell, Athens, Greece). Firstly, endogenous nitrite was measured colorimetrically by detection of an azo dye product of the Griess reaction. Secondly, nitrate was converted to nitrite by nitrate reductase and the total nitrite concentration was determined. Nitrate concentration was calculated by subtraction of endogenous nitrite from total nitrite.

iNOS concentration in plasma was measured by means of a commercial enzyme-linked immunoassay (ELISA) kit following the instructions of the manufacturer (CUSABIO BIOTECH CO., LTD, Hubei Province, PRC). The method was based on the incubation of diluted plasma samples and standards on anti-iNOS antibody pre-coated microplate wells, and the subsequent detection of bound antigen - antibody complexes by a biotin-conjugated antibody specific for iNOS (sandwich ELISA). For visualization of the reaction, an avidin-conjugated horseradish peroxidase was added, converting a chromogenic substrate to a photometrically measureable product, determined using a microplate reader set to 450 nm with wavelength correction set to 570 nm. The minimum detectable dose of human iNOS was typically less than 0.225 IU/ml.

Statistical analysis was conducted using SPSS version 15.0 (SPSS Inc., Chicago IL, USA) and Stata version 9.2 (Statacorp LP, College Station, TX, USA). Extremely skewed parameters were log-transformed to achieve normality. Fisher’s exact test was used for comparisons of categorical data. ANOVA was used, with post-hoc Bonferroni-corrected method for multiple comparisons when needed, to univariately compare mean values between (controls, patients) and within (three discrete time-points of measurement) groups, including all participants. All statistical inferences were based on two-tail probabilities. Statistical significance was set at the p-value of less than 0.05.

**Results**

**Endogenous nitrite, total nitrite and iNOS concentrations.** The mean values for all three parameters were significantly higher in alcohol-dependent individuals compared to controls at all times of measurement (p<0.001, Table I). Significantly higher endogenous nitrite value was only found at discharge vs. admission (p=0.031).

Endogenous and total nitrite concentrations were found to be positively correlated (p=0.001, results not shown), being diagnostically equally conclusive. iNOS values were not correlated with the other two study parameters (iNOS vs. endogenous nitrite p=0.311; iNOS vs. total nitrite p=0.384, results not shown).
Finally, in the control group, total nitrite and iNOS concentrations were significantly higher in men than in women (for total nitrite \( p=0.048 \) and for iNOS \( p<0.001 \), results not shown).

**Routine laboratory examinations.** A significant decline of MCV, SGOT, SGPT and \( \gamma \)GT values, which routinely serve as indices of the patient’s alcohol misuse, was observed; this is consistent with what is usually expected during alcohol detoxification. There were no significant differences in glucose and TSH concentrations between patients and the control group (Table I).

### Discussion

ALD is an inflammatory condition resulting from a variety of factors, including ethanol-induced metabolic-associated oxidative stress glutathione depletion, abnormal methionine metabolism, malnutrition and activation of innate immunity (25, 26). Lipopolysaccharide, complement, and tumor necrosis factor-\( \alpha \), a pro-inflammatory cytokine, play an important role in initiating and promoting ALD. Concurrently, anti-inflammatory and hepatoprotective cytokines, such as interleukin-6 (IL6), IL10, IL22 and adiponectin are up-regulated, as a compensatory mechanism protecting against alcoholic injury and inflammation (27-30). Ethanol consumption inhibits the function of natural killer cells, which play key roles in anti-viral, antitumor and antifibrotic defense in the liver (31, 32).

In vitro studies have shown that alcohol-induced intestinal barrier disruption is caused by the action of iNOS, through NO overproduction, leading to oxidative tissue damage, gut leakiness, endotoxemia and liver injury (11). iNOS is also responsible for SNAIL activation, stimulating EMT, resulting in the disruption of intestinal cell permeability (15). In alcohol abuse, on the clinical level, iNOS determination, either directly or through endogenous nitrite/total nitrite determination, might serve as a prognostic marker for NO-dependent gut-associated ALD pathogenesis.

The population of the present study comprised of individuals without liver disease, who were hospitalized for a four- to six-week detoxification therapy. Determination of
endogenous nitrite, total nitrate and iNOS concentrations at three time points, i.e. upon admission, two weeks after, and before discharge, showed that all three parameters were significantly higher than in the control population during the entire treatment. No trend for a decrease was observed, even for those with concentrations higher than of the controls between admission and discharge, in contrast to the other routine laboratory examinations. In a previous report, specific markers for alcohol overconsumption, such as carbohydrate-deficient transferrin and IL6, were found to drop significantly following this four- to six-week detoxification program (24).

Obviously, it is practically difficult to follow-up the course of iNOS changes after completion of detoxification and patient discharge; thus, it is unknown whether and when the enzyme concentrations may reach normal levels. Taking into consideration the pathogenetic involvement of iNOS in ALD, we may hypothesize that the increase in enzyme concentration and activity are already established in alcohol dependence before the onset of ALD. Although alcohol-dependent individuals without liver disease could be considered as a group at high-risk for development of ALD if untreated, it is problematic for ethical reasons to run studies in order to acquire data regarding the association of alcohol abuse and onset of ALD.

The present clinical study is in agreement with previous experimental results showing the positive pathogenetic relationship between iNOS and alcohol abuse (11, 12, 15). The main challenge arising would be the preventive therapeutic use of iNOS inhibitors in the present population in order to avoid the injurious effects of chronic alcohol abuse. In experimental animal models, the non-selective L-arginine analog NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME), which is active against all NOS isoforms, and the selective iNOS inhibitor L-N6-(1-iminoethyl)-lysine (L-NIL) have been effective in preventing the manifestation of ALD (11, 12). In previous reports, both agents have been clinically implemented in humans, L-NIL for the treatment of asthmatic patients and L-NAME in cardiovascular disorders (33, 34). Their potential use in alcohol-dependent individuals for the prevention of oxidative stress-induced intestinal hyperpermeability and endotoxiaemia, as well as liver injury, should be a matter for further experimental and clinical investigation.

References


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