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ORIGINAL ARTICLE

Serum MDA, Antioxidant Vitamins and Erythrocytic Antioxidant Enzymes in Chronic Alcoholic Liver Disease – A Case Control Study

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Abstract: *Objectives:* The study aims to estimate the changes in the serum levels of lipid peroxidation product malondialdehyde (MDA), non-enzymatic antioxidants: vitamin A, E and C and erythrocyte enzymatic antioxidants: superoxide dismutase (SOD) and catalase(CAT) in chronic alcoholic liver disease. *Background:* Alcohol consumption accounts for about 50% of patients death from end stage liver disease in India. The increased free radical and their metabolites decrease the plasma antioxidants status in chronic alcoholic liver disease (CALD). *Method:* The study comprised of 100 healthy persons as controls and 100 diagnosed patients of chronic alcoholic liver disease as cases. The estimation of serum MDA, vitamin A, E, C and erythrocyte enzymatic antioxidants SOD and CAT, were carried out along with liver function parameters like serum aspartate amino transferase (AST), serum alanine aminotransferase (ALT), serum alkaline phosphatase (AP), serum gamma glutamyl transferase (GGT), serum total protein, serum albumin, prothrombin time (PT) and serum bilirubin. Statistical analysis was done using unpaired "t" test. *Result:* The levels of serum MDA were significantly increased in patients with CALD ($P<0.01$) while antioxidants were significantly reduced as compared to controls ($P<0.01$). *Conclusion:* Increased levels of lipid peroxides and reduced antioxidants suggest that, oxidative stress plays a vital role in pathogenesis of chronic alcoholic liver disease.

Key words: Chronic alcoholic liver disease, serum MDA, serum antioxidant vitamins, erythrocytic SOD/CAT.

Introduction

Liver diseases due to excessive alcohol consumption are a major public health problem leading to morbidity and mortality throughout the world [1]. The prevalence of alcohol intake is reported as 5-20% in India [2]. The liver quite rightly has been called the "custodian of the milieu interior" metabolizes almost all ingested alcohol. Oxidative stress is a disturbance in the oxidant-antioxidant balance leading to potential cellular damage. The imbalance can result from a lack of antioxidant capacity caused by disturbance in the production and distribution, or by an abundance which can damage lipids, proteins and DNA inhibiting normal function [3]. Alcohol metabolism generates highly reactive molecular fragments called reactive oxygen species (ROS). The ROS can diffuse from the site of generation and damage the structural and functional integrity of cells causing tissue damage [4].

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The peroxidation of lipid components of the cells by ROS generates toxic species like lipid peroxides, lipid hydroperoxides and aldehyde breakdown products [5]. Increased ROS production lowers cellular antioxidant levels and enhances the oxidative stress in many tissues, especially liver [3]. Alcohol consumption is associated with a number of changes in cell functions and the oxidant-antioxidant system [6]. Vitamin C (ascorbate) reacts rapidly with superoxide, peroxyl and hydroxyl radicals to give semidehydroascorbate [7]. Vitamin A and Vitamin E are lipid soluble, chain breaking antioxidants and serve as the first line of defence against peroxidation of membrane lipids [8]. Superoxide dismutase (SOD), one of the important intracellular antioxidant enzyme present in aerobic cells has antitoxic effect against superoxide radical [9]. Catalase (CAT) protects the cells from accumulation of H_20_2 by decomposing it to H_20 and 0_2 [10]. Malondialdehyde (MDA) is the end product of the lipid peroxidation, while SOD, CAT and other antioxidants are involved in the elimination of free radicals. They are assumed to represent pro-oxidant and antioxidant factors respectively in the cellular free radical metabolism. The balance of these two decide the net result of cellular and/or tissue oxidation/peroxidation state. In this context, the study was undertaken to determine the changes in the serum levels of MDA, vitamin A, Vitamin C and Vitamin E and erythrocytic antioxidant enzymes SOD and CAT in chronic alcoholic liver disease (CALD) patients.

Material and Methods

The present study was carried out jointly by the department of Biochemistry and General medicine in S. Nijalingappa Medical College and Hanagal Shri Kumareshwara (HSK) Hospital and Research Centre, Bagalkot , from June-2007 to July-2008. Institutional ethical committee approved the study and written informed consent was taken from all the subjects. Properly matched 200 subjects were selected for the study, comprising 100 healthy controls from Bagalkot city and 100 diagnosed cases of chronic alcoholic liver disease (CALD) admitted to HSK hospital, Bagalkot. All subjects were males. Detailed medical history was obtained and physical examination was done for all the subjects. Ethanol intake was assessed by recall method and calculated by using the formula: Grams of alcohol = volume of beverage x strength (V/V, as %) x 0.8 [11].

Inclusion Criteria: Patients attending medicine department on OPD/IPD basis with history of alcohol intake for more than ten years, with a daily intake of about 42.5±3.15 grams and with complaints regarding tender hepatomegaly/ jaundice/ ascites.

Exclusion Criteria: Subjects with diabetes mellitus, renal disorders, hypertension, drug induced hepatitis, smokers and non alcoholic liver disease of any etiology were excluded. Patients with HIV, hepatitis B and hepatitis C diagnosed by tridot and confirmed by ELISA, were also excluded from the study. Liver disease patients with unknown etiology were also excluded.

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Sample collection and estimation: Fasting venous blood was collected in two test tubes, one heparinised tube and another plane tube, under aseptic precautions and analyzed for the study parameters. Serum was used for MDA estimation, antioxidant vitamins - vitamin A (Retinol), vitamin C (Ascorbic acid) , vitamin E (α-tocopherol), aspartate amino transferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), gamma glutamyl transferase(GGT), total protein, albumin, prothrombin time (PT), total bilirubin and conjugated bilirubin . MDA was measured by Thiobarbituric acid reactive substances assay (TBRAS) method [12], vitamin A was estimated by Bessey method [9-10], vitamin C by Dinitrophenyl Hydrazine (DNPH) method [13] and vitamin E by Quaife method [14]. The optical densities of MDA and vitamin A were measured at 532 nm, 327nm respectively. Vitamin C and Vitamin E were measured at 520nm using spectrophotometer. GGT is one of the sensitive parameter for alcoholic liver disease was estimated by modified colorimetric method of Szasz [15] (kit supplied by Riachem), AST (Riachem), ALT (Riachem), AP (Riachem), Total protein (Span diagnostics), albumin (Span diagnostics), total bilirubin (ERBA) and conjugated bilirubin(ERBA) were estimated by kit method using semi automated analyzer Statfax 3300. Prothrombin time was measured manually by using kit supplied by Tulip diagnostics (P) Ltd. The antioxidant enzyme activities of erythrocytic SOD and CAT were measured in appropriately diluted haemolysates. To prepare the haemolysate, RBCs were first isolated by passing the blood through L- cellulose and microcrystalline cellulose column [16]. The cells were re-suspended in a stabilizing medium (comprising of 2.7mM EDTA and 0.7mM β-mercaptoethanol) and lysed by freeze–thaw technique [17]. The SOD activity was measured in chloroform-ethanol extract of haemolysate based on its ability to inhibit auto-oxidation of epinephrine to adrenochrome at pH 10.2 [18]. The CAT activity was determined spectrophotometrically by noting the decline in optical density at 230 nm of decomposition of Hydrogen peroxide H_20_2 [19]. The specific activity of enzymes were expressed in International unit per gram of hemoglobin (IU/gHb). The hemoglobin level was estimated by using cyanometh reagent [20].

Statistical analysis: Results of the study were analyzed by unpaired 't' test. P value less than 0.05 were considered statistically significant.

Results

Table 1 depicts characteristics of CALD cases and controls. There is significant elevation ($p<0.01$) of ALT, AST, AP, GGT, total bilirubin, conjugated bilirubin and unconjugated bilirubin in CALD compared to control group. There is a significant decrease ($p<0.01$) in serum total protein and albumin in CALD than controls. There was no significant decrease in PT in CALD compared to control subjects.

Table 2 shows serum MDA, antioxidant vitamins - A, E and C, erythrocytic SOD and CAT levels of CALD patients and healthy controls. Significantly increased ($p<0.01$) level of MDA and significantly decreased ($p<0.01$) levels of serum antioxidant vitamins (A, E and C) and erythrocytic enzymatic antioxidants (SOD and CAT) were noticed in CALD as compared to controls ($P<0.01$).

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Table-1: Characteristics of study subjects		
Parameters	CALD (n=100) cases	Controls (100)
Number	100	100
Age (Years)	38±6	39±6**
BMI (kg/ m^2)	24.7±2.6	26.3±3.4***
Ethanol consumption(gm)	42.25±3.15	--
Serum AST (U/L)	86.4±24.66	13.34±7.30*
Serum ALT(U/L)	49.9±10.59	14.10±7.49*
Serum AP(U/L)	129.13±25.61	40.11±24.40*
Serum GGT (IU/L)	124.28±25.00	12.75±6.73*
Serum total protein (g/dl)	6.01±0.71	6.98±0.58*
Serum albumin (g/dl)	3.48±0.0.49	3.88±0.46*
Prothrombin time (Seconds)	14.5±1.7	13.5±1.5**
Serum total bilirubin (mg/dl)	2.93±0.75	0.76±0.32*
Serum unconjugated bilirubin(mg/dl)	1.53±0.51	0.54±0.21*
Serum conjugated bilirubin (mg/dl)	1.40±0.24	0.22±0.06*
Values are expressed as Mean ± SD, *p<0.01, statistically significant., **p>0.05 statistically not significant., *** p<0.005 statistically significant., CALD: Chronic alcoholic liver disease, BMI: Body mass index., AST: Aspartate amino transferase, ALT: Alanine aminotransferase., AP: Alkaline phosphatase. GGT: Gamma glutamyl transferase		

Table-2: Comparison of malondialdehyde and antioxidant status in CALD and control groups		
Parameters	CALD (n=100) cases	Controls(n=100)
Serum MDA (n mol/ml)	7.02±0.96	1.97 ± 0.66*
Erythrocyte SOD (IU/g Hb)	587.22±190.96	739.74 ± 154.88*
Erythrocyte CAT (IU/g Hb)	7.22±1.86	8.50±2.22*
Serum Vitamin A (mg/dl)	41.26±9.72	90.81±33.54*
Serum Vitamin C (mg/dl)	0.87±0.31	1.54±0.32*
Serum Vitamin E (mg/dl)	0.59±0.21	1.57±0.36*
Values are expressed as Mean ± SD., * p<0.01, statistically significant change., CALD: Chronic alcoholic liver disease., MDA: Malondialdehyde., SOD: Superoxide Dismutase, CAT: Catalase,		

Discussion

In the present study, serum MDA, the marker of oxidative degradation is significantly increased; significant decrease in levels of serum antioxidant vitamins (A, E and C) and enzymatic antioxidants (Erythrocytic SOD and CAT) were noticed in CALD as compared to controls (P<0.01). Many studies have shown similar results, ie, significant increase in serum MDA and significant decrease in serum levels of vitamins A, C and E and erythrocytic CAT [21-24]. But, there is controversy over decreased erythrocytic SOD and chronic alcoholic liver disease. Our results are in accordance with Liala Guemouri et al [25], Sumit Bhandri. [22]. Virgina and co-workers in their rat experiment found that there was no significant change in CAT and vitamin E levels in hepatic tissue of ethanol fed group and control group [26] . Several pathways have been suggested as to how chronic ethanol consumption leads to increased oxidative stress.

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Ethanol is metabolized to acetaldehyde by alcohol dehydrogenase in liver and acetaldehyde is oxidized to acetate by aldehyde oxidase or xanthine oxidase giving rise to ROS via cytochrome P450 [27]. Ethanol is a hydroxyl radical scavenger; the product of the interaction of ethanol with hydroxyl radical is 1 hydroxyl ethyl radical [HER]. Liver microsomes can oxidize ethanol to HER in an NADPH or NADH dependent manner by iron catalyzed process [24, 28]. Production of reactive product, acetaldehyde, by ethanol effects on the immune system and altered cytokine production, ethanol induction of Cytochrome P450 2E1 (CYP2E1), antioxidant enzymes and chemicals, particularly mitochondrial and cytosolic glutathione; oxidation of ethanol to the 1-hydroxy ethyl radical [28]. Reactive aldehydes produced from ethanol and ethanol induced oxidant stress such as acetaldehyde, MDA and 4-hydroxy-2nonenal (HNE), and HER can bind to proteins to produce protein adducts. MDA acetaldehyde adduct can bind to other proteins to produce hybrid adducts. Such adducts can produce toxicity because the adducted proteins may loose function and provoke immune response. ROS interact with antioxidants and inactivates antioxidant enzymes, producing a state of oxidative stress [28].

Chronic ethanol treatment elevates endotoxin level, endotoxin activates kupfer's cells to produce free radicals via NADPH oxidase. The free radicals activate nuclear factor –kappa B (NF-kB), leading to an increase in production of tumor necrosis factor alfa (TNFα), followed eventually by tissue damage [28]. Endotoxin is one of the component of the outer cell wall of gram negative bacteria. The mechanism by which ethanol activates kupfer's cells by endotoxin is not clear, but could involve alteration of gut permeability to endotoxin, modification of gut flora or changes in rates of endotoxin clearance [29]. Acetaldehyde, produced by the oxidation of alcohol, is able to inhibit the repair of alkylated nucleoproteins, to decrease the activity of several enzymes and to damage mitochondria [3]. Alcoholic hepatitis is considered as an inflammatory condition. Pro inflammatory cytokines such as TNFα and interleukin 6 (IL-6) are directly involved in the pathogenesis. Cytokines released by these cells initiate the cascade of events, which ultimately leads to the development of hepatic necrosis, recruitment of neutrophils and immune cells, and liver fibrosis [24]. Lipid peroxides stimulate stellate cells, which are major source of extracellular matrix, thus stimulating collagen synthesis, which may be important in many forms of liver fibrosis [23].

The reduced levels of vitamin A in alcoholics is due to exhaustion of hepatic storage by enhanced ROS, hence reduced conversion of β-carotene to vitamin A and reduced release of vitamin A from liver into circulation. Chronic alcoholism may also interfere with retinoid metabolism and enhance microsomal degradation of vitamin A. [30]. Ascorbate is reduced in chronic alcoholic liver disease patients because it is used to regenerate α–tocopherol from α–tocopheroxyl radical at water-lipid interface. It is also an efficient quencher of superoxide and hydroxyl radicals. Significant decrease of plasma α–tocopherol in CALD may be due to enhanced lipid peroxidation by alcohol metabolism. Alcohol may also affect the pharmacokinetics and distribution of α–tocopherol [31]. In addition to the above, there may be associated nutritional deficiency [32].

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Diminution of SOD and CAT activity in chronic alcoholic liver disease patients could be in relation to increased exposure to oxidant environment that can destabilize RBC membrane by lipid peroxidation and cause significant leakage of these intracellular enzymes. There are evidences for plasma membrane becoming leaky owing to extensive damage by peroxidative attack which allows leakage of cytosolic enzymes from whole cells [33]. Oxidative inactivation of the enzymes is also possible. Further increased alcohol consumption leads to poor dietary intake of biometals adversely affecting the activity of these metalloenzymes. Superoxide dismutase may be useful tool for the assessment of disease severity and monitoring drug therapy [34]. The decrease in the activity of catalase could be due to increase in malondialdehyde (MDA) which can cross-link with amino group of protein to form intra and intermolecular cross-links thereby inactivating several membrane bound enzymes [35].

The drawback of this study was correlation between the serum MDA, erythrocytic SOD, CAT and hepatocyte MDA, SOD, CAT was not done, which could have strongly substantiated the role of oxidative stress and antioxidants in alcoholic liver disease. One limitation for this study was that there were obvious contraindications for liver biopsy in most of the CALD patients.
Further studies and invasive techniques like liver biopsy is needed, so that correlation between the serum levels of MDA, erythrocytic SOD, CAT and liver MDA, SOD, CAT levels can be established.

Conclusion

In conclusion, the present study is consistent with previous studies suggesting that oxidative stress appears to be of immense value in understanding the pathogenesis of alcoholic liver disease. The serum MDA can be considered as one of the sensitive markers of liver damage by alcohol. In alcoholic liver disease, serum antioxidants and erythrocytic antioxidant enzymes may be utilized to a greater extent to counteract free radical mediated cellular changes, resulting in the reduction of plasma antioxidant levels.

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