Indian Journal of Clinical Biochemistry, 2005, 20 (2) 24-28

MONITORING OXIDATIVE STRESS IN PATIENTS WITH NON-ALCOHOLIC AND ALCOHOLIC LIVER DISEASES

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ABSTRACT

Ethanol- induced liver injury may be linked, at least partly, to an oxidative stress resulting from increased free radical production and/ or decreased antioxidant defence. Distinguishing alcoholic and non-alcoholic liver disease has important implications. This study looked at the possible changes between alcoholic and non-alcoholic liver diseases by examining the presence of oxidative damage, as monitored by several parameters relating to oxidative stress. Lipid peroxides concentration, superoxide dismutase activity and glutathione S-transferase activity increased, where as glutathione content, glutathione peroxidase activity and glutathione reductase activity decreased among the tested subjects in comparison to normal healthy group. Determination of these parameters may be valuable in the evaluation of liver disease. However, oxidative stress related enzymes and non-enzymes can not be utilized as a marker for alcoholic liver diseases, as these parameters responded in the same way after liver is damaged irrespective of their cause. Their level may help in determining the degree of liver damage. Degree of oxidative injury was similar in patients with non-alcoholic liver disease and in moderate drinkers; while significantly higher in heavy drinkers. The differences between the groups might be based on the type of liver pathological condition rather than its etiology (i.e. alcohol and non alcohol related causes).

KEY WORDS

Alcohol, Oxidative stress, Glutathione, Superoxide dismutase, Lipid peroxide.

INTRODUCTION

Oxidative stress is well recognized to be a key step in the pathogenesis of ethanol-associated liver injury. An imbalance between oxidants and antioxidants can lead to oxidative stress, characterized by escalating cell damage (1). The metabolic effects of alcohol are due both to its direct action and to that of its first metabolite acetaldehyde, and can also be connected with the changes in redox state. Differences in ethanol distribution, bioavailability and hepatic metabolism can provide insight into the protective and predisposing factors in alcoholism. Oxidative stress occurs following various conditions of ethanol consumption. Alcoholic dependence causes gradual exhaustion of the antioxidant capacity of erythrocytes, therefore this non-invasive measurement may be useful as a follow-up of the alcoholic liver disease (2).

Toxic substances generated during the metabolism of alcohol in the liver may contribute to the development of alcoholic liver disease. These substances include highly reactive molecules that can destroy vital cell components through a process called oxidation (1, 3). Alcoholic liver diseases may be caused by oxygen radicals such as superoxide and hydroxyl radicals, generated during the metabolism of ethanol by the microsomal oxidising system (4).

In the present study, stress related enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione s-transferase (GST), and glutathione reductase (GR); and antioxidants like ascorbic acid and reduced glutathione content were measured and compared in patients with liver diseases either due to alcohol or without alcohol with a group of normal healthy persons.

MATERIALS AND METHODS

Selection of Subjects

A total of 181 subjects were studied: 81subjects (43

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males and 38 females) with alcoholic liver disease (ALD); 45 patients (24 males and 21 females) with non-alcoholic liver disease (NALD); and 55 normal healthy presenters (33 males and 22 females) without any reported disease. Alcoholic patients were further classified as described by Paton (5) into: a) High alcohol intake group (ALD-H; those had been drinking more than 80g alcohol per day for at least one year), and b) Moderate alcohol intake group (ALD-M; those had been drinking less than 80g alcohol per day). 24 male and 22 female were in High alcohol intake group; while 19 male and 16 female were in Moderate alcohol intake group. These patients were selected from those who had visited Central Referral Hospital, Gangtok; and categorized on the basis of oral questionnaires and laboratory findings. Consent was obtained from every subject.

All chemicals used in this study were of analytical grade.

Methods

Total protein in serum was determined by biuret method using potassium sodium tartrate reagent and alkaline copper sulphate solution (6). Hemoglobin content was estimated using cyanmeth reagent (7). Ascorbic acid content was determined using dinitrophenylhydrazine- thiourea- copper sulfate reagent at 520 nm as described by McCormick and Greene (8).

Whole blood was collected by venepuncture in heparinised vials from subjects. 1.5ml of heparinised whole blood was centrifuged at 1000g for 10 minutes at 2°C. Plasma was separated. Packed erythrocyte was washed with 4.0 ml cold normal saline thrice and in each case centrifuged at 15000g for 15 minutes at 2°C. Washed, packed erythrocyte was hemolysed by the addition of 30 ml chilled double distilled water. The hemolysate was centrifuged at 13000g for 1 hour at 2°C. The washed centrifuged erythrocytes were then used to assay glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PD), superoxide dismutase (SOD), glutathione (GSH), catalase content, and lipid peroxidation (TBAR).

Thiobarbituric acid reactive (TBAR) substance of the sample was determined using TCA and thiobarbituric acid (TBA) as described by Sinnhuber *et al.* (9). Glutathione content was estimated according to the method of Beutler *et al.* (10) using DTNB reagent [5,5'-dithiobis(2-nitrobenzoic acid)]. The yellow colored product was read at 412 nm. Catalase (EC 1.11.1.6) activity of the sample was determined using the sample as enzyme source and H_2O_2 in phosphate buffer. The change in absorbance was read at 240 nm. The specific activity was calculated using molar extinction coefficient 43.6 M^{-1} cm^{-1} for H_2O_2 at 240 nm (11).

Glutathione reductase (GR; EC 1.6.4.2) was assayed by the method of Pinto and Bartley, (12). The enzyme activity was quantified by measuring the change in absorbance at 340 nm using the extinction coefficient for NADPH of 6.22 $cm^{-1}/\mu M$. The activity of Glutathione peroxidase (EC 1.11.1.9) was assayed according to the method of Paglia and Valentine (13). Glutathione S-transferase (GST) (EC 2.5.1.18) was measured by the method of Habig *et al.* (14) using CDNB (1-chloro-2, 4-dinitrobenzene) reagent. The Superoxide dismutase (EC 1.15.1.1) activity was assayed

Table 1. Ascorbic acid, thiobarbituric acid reactive substance (TBARS), and reduced glutathione (GSH) content of normal healthy persons, and patients with alcoholic liver disease and non alcoholic liver disease.

Group	Ascorbic acid (mg/dl)	TBARS (nmol/ml)	GSH content (µg/ mg protein)
Normal Healthy Persons (n=55)	1.28 ± 0.17	3.43 ± 0.23	3.58 ± 0.25
Non Alcoholic Liver Disease (n=45)	1.12 ± 0.16	8.7 ± 0.47*	2.93 ± 0.13*
Alcoholic Liver Disease (Moderate Alcohol Intake) (n=35)	0.94 ± 0.11	6.36 ± 0.29*#	3.16 ± 0.16
Alcoholic Liver Disease (High Alcohol Intake) (n=46)	0.75 ± 0.09*#	11.7 ± 0.67*#@	2.72 ± 0.15*@

Values are mean ± SEM of number of observations (n). * indicates $p < 0.05$ when compared with normal healthy control # indicates $p<0.05$ when compared with non alcoholic liver disease and @ indicates $p<0.05$ when compared with alcoholic liver disease with moderate alcohol intake.

Table 2. Superoxide dismutase, catalase, glucose-6-phosphate dehydrogenase glutathione reductase, glutathione peroxidase and glutathione S-transferase, activity in normal heathy persons, alcoholoic liver disease and non-alcoholic liver disease groups

	Normal Healthy Persons (n=55)	Non Alcoholic Liver Disease (n=45)	Alcoholic Liver Disease (Moderate Alcohol Intake) (n=35)	Alcoholic Liver Disease (High Alcohol Intake) (n=46)
Superoxide dismutase[a]	1.24 ± 0.02	1.49 ± 0.11*	1.62 ± 0.03*#	2.04 ± 0.04*#@
Catalase[b]	0.136± 0.005	0.134 ± 0.002	0.133 ± 0.002	0.124 ± 0.004*
Glucose 6 phosphate dehydrogenasec	7.84 ± 0.05	7.81 ± 0.04	7.63 ± 0.05*#	6.8 ± 0.16*#@
Glutathione reductase[d]	1.814± 0.008	1.684 ± 0.005*	1.652 ± 0.013*	1.574 ± 0.029*@
Glutathione peroxidase[e]	52.46 ± 1.36	31.56 ± 1.3*	35.46 ± 1.14*#	23.54 ± 0.32*#@
Glutathione s- transferase[f]	1.11 ± 0.01	1.18 ± 0.03*	1.21 ± 0.02*	1.61 ± 0.1*#@

a: U/ mg haemoglobin; b: mmol H_2O_2 decomposed/ mg protein / min; c: U/ g haemoglobin; d: U/ mg haemoglobin; e: U/ g haemoglobin; f: nmol CDNB conjugate formed/ min/ mg protein.

*Values are mean ± SEM of number of observations (n). * indicates p < 0.05 when compared with normal healthy control # indicates p<0.05 when compared with non alcoholic liver disease and @ indicates p<0.05 when compared with alcoholic liver disease with moderate alcohol intake.*

according to the method of Paoletti *et al* (15) by measuring the oxidation of NADH; and Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was assayed as described by Kornberg and Horecker (16).

Results have been expressed as mean±SEM (standard error). Statistical significance was determined by Student's 't' test for unpaired data. The values of significance were evaluated with 'p' values. The difference were considered significant at $p<0.05$.

RESULT

Ascorbic acid, thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) content of the entire groups were presented in Table 1. Only high alcohol intake group showed significant decrease in ascorbic acid content, whereas the glutathione levels of non-alcoholic liver disease and High alcohol intake groups showed significant decrease in comparison to normal healthy persons. Significant increases in TBARS content in all the study groups were observed when compared with the normal healthy group (Table 1).

Superoxide dismuatase activity increased in alcoholics compared to normal healthy group. On the other hand, the catalase activity of heavy alcohol intake group; and glucose-6-phosphate dehydrogenase activity of both the alcoholic liver disease groups significantly decreased in comparison to other groups (Table 2). Significant decline in glutathione reductase and glutathione peroxidase activities and significant rise in glutathione-S-transferase activity were observed in all the tested groups in comparison to normal healthy individuals (Table 2).

DISCUSSION

Alcohol consumption is associated with a number of changes in cell functions and the oxidant-antioxidant system (17). Patients with alcoholic liver diseases due to high alcohol consumption showed significant decrease in ascorbic acid content whereas patients with various forms of liver disorders showed increased levels of lipid peroxides as determined by thiobarbituric acid reactive substances (TBARS). Olinescu *et al.* (18) suggested that in severely impaired liver, lipid peroxides might be also produced by a mechanism involving heme oxygenase. Reduced antioxidant capacity has been found in several tissues and may promote the generation of free radicals and lipid peroxides. Lipid peroxidation mediated by free radicals is considered to be a primary mechanism of cell membrane destruction and cell damage (19). Glutathione (GSH) plays a major role in cellular protection against oxidative damage. Patients suffering from liver diseases either due to non-alcohol or excessive alcohol intake showed depletion of GSH level. Several factors contribute to the fall in GSH level in alcoholic and non-alcoholic liver diseases. Most important is oxidative stress, which occured in this study and consumes GSH. Depletion of GSH renders the cell more susceptible to oxidative stress (20).

In addition to being a direct free radical scavenger, GSH is known to function as a substrate of glutathione peroxidase (GPX) and glutathione S-transferases (GST) (21). Decrease in the activity of glutathione peroxidase in the present investigation may be due

to exhaustion or inactivation of the enzyme by reactive oxygen species, since oxidative damage to haemoglobin and cell membrane has been reported to reduce the activity of glutathione peroxidase (22, 23, 24). Initial oxidation of the GSH pool results in the formation of the GSSG by H_2O_2 and glutathione peroxidase. A subsequent interaction of the free cysteine thiol of the active protein tyrosine phosphatase (PTP) with GSSG forms the mixed disulfide and concomitant inactivation of the enzyme. A competing rate results in the restoration of the GSH pool from the GSSG by the interaction with glutathione reductase and NADPH. It is important to note that an inefficient rate of GSH restoration, as in the case in conditions of high oxidative stress, can result in levels of GSSG that will result in an accelerated or continued state of phosphatase inactivation (25). Decreased GR activity may be a predominant cause for GSH depletion within the RBC leading to serious consequences like increased lipid peroxidation and hemolysis. Glutathione s-transferase (GST) plays an essential role in liver by eliminating toxic compounds by conjugating them with glutathione. The activities of glutathione reductase and glutathione s-transferase these two enzymes are found to be in reverse order.

The increase in erythrocyte SOD activity may probably be an adaptive response towards oxidative stress (26). Thome *et al.* (27) observed that patients suffering from alcohol dependence the mean SOD concentration reached almost double the values of those from the non-dependent healthy subjects. Superoxide dismutase may be useful tool for the assessment of disease severity of monitoring drug therapy (28). Specifically, over expression of SOD results in increased dismutation of superoxide to H_2O_2.

Significant decrease in the activity of catalase could be due to less availability of NADPH. However, the gradual decrease in erythrocyte catalase activity may be due to excessive generation of $O_2^{\cdot-}$ leading to inactivation of the enzyme, as $O_2^{\cdot-}$ has been shown to reduce catalase activity (26). Our study showed a significant decrease in the activity of catalase in alcoholic liver diseases. This 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 (29).

From the present study, it is very difficult to distinguish between the patients suffering from liver diseases either due to non-alcohol or moderate alcohol intake. But alcoholic patients who were consuming high amount of alcohol are suffering from severe liver damage. Oxidative stress related enzymes and non-enzymes responded in the same way after liver is damaged. None of these parameters can be utilised as a marker for alcoholic liver disease. Antioxidants and stress related enzymes might be able to determine the degree of liver damage. The differences between the groups might be based on the type of liver pathological condition rather than its etiology (i.e. alcohol and non-alcohol related causes).

ACKNOWLEDGEMENT

Financial assistance received from Council of Scientific and Industrial Research (CSIR) is gratefully acknowledged.

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