Free Radical Scavenging Enzymes, Activities and their Correlation with Malondialdehyde in Actue Infective Hepatitis Patients

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SUMMARY

In this study, we investigated erythrocyte superoxide dismutase (SOD) and catalase (CAT) activities as antioxidant enzymes and malondialdehyde (MDA) as a sign of lipid peroxidation in AIH patients, along with routine parameters of liver disease. Present investigations were carried out to evaluate these special parameters in patients with clinical history of AIH and to assess the utility of these parameters as diagnostic/ prognostic indices of liver function and to correlate with special parameters with routine liver function tests (LFT). SOD, CAT, and MDA along with routine LFT was estimated in 25 patients with AIH and 25 samples from healthy voluntary blood donors served as control group. Routine LFT was estimated by standard clinical chemistry procedure on autoanalyzer and SOD, CAT and MDA were estimated by NBT reduction, H_20_2 reduction, and TBA method, respectively. In patient group, erythrocyte SOD and CAT activities were strongly positive correlated with MDA concentration. These findings also provide a theoretical basis for the development of novel therapeutic strategies such as supplementation of certain specific enzymatic and/or non enzymatic antioxidants.

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INTRODUCTION

In human, different organs play certain roles in metabolic processes. Each organ carries out functions which are specific. In order to assess qualitatively and quantitatively the capacity of the tissue of a particular organ to carry out the functions, organ function tests are devised. Most of these tests are measurements of blood components which provide an insight into the extent and type of tissue damage. More sophisticated methods may need to be done in employed in order to pinpoint the damage. The major organ function tests are those of liver, kidney and thyroid.

As far as liver is concerned, it is a major organ playing a central role in the body due to its anatomical connections and biochemical functions. It is of vital significance in intermediary metabolism and in detoxification and removal of various toxicants [1,2]. Liver is the only organ that possesses the enormous capability of regeneration in human. In general, damage to the organ may not obviously affect its activity. Since the liver has considerable functional reserve and as a result, simple liver function tests like serum albumin and bilirubin concentrations are insensitive indicators of liver diseases [3]. The results obtained from biochemical tests are not frequently specific, as they show the fundamental pathological processes common to various diseased conditions, and therefore provide limited diagnostic information. That is why tests reflecting liver cell damage particularly monitoring the activities of certain hepatic enzymes in serum or plasma are often superior in this regard.

In fact, liver disease is accompanied by an increased production of free radicals [3]. Hepatocyte membranes are quite rich in PUF A, which are susceptible to attack by free radicals. The PUFA of the membrane undergo peroxidation. Free radicals, primarily the reactive oxygen species, superoxide and hydroxyl radicals which are highly reactive having an impaired electron in an atomic or molecular orbit are generated under physiological conditions during aerobic metabolism. As free radicals are potentially toxic, they are usually inactivated or scavenged by antioxidants before they can inflict damage to lipids, proteins or nucleic acids [4]. The human body has a complex antioxidant defense system that includes certain antioxidant enzymes such as superoxide dismutase (SOD) and the catalase (CAT). These block the initiation of free radical chain reactions [5,6]. However, when free radicals are produced in excess or when the cellular antioxidant defense system is defective, they can stimulate chain reactions by interacting with proteins, lipids and nucleic acids causing cellular dysfunction and even death. Since the AIH patients are under oxidative stress which has exhausted the ability of their antioxidative capacity to adapt to the elevated levels of circulating peroxides, we decided to estimate the level of lipid peroxidation product (MDA) and the activities of antioxidative enzymes, SOD and CAT. The principal aim of the present study is to evaluate these parameters in patients with clinical history of AIH and the objective is to assess the utility of these parameters as diagnostic/prognostic tools of liver

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function and to correlate between these parameters and routine LFT.

MATERIALS AND METHODS

This study was performed on blood samples from 30 patients of age group ranging from 20 to 60 years with acute infective hepatitis sent for investigations to diagnostic clinical laboratory at SRMS Institute of Medical Sciences, Bareilly. Patients with other diseases such as diabetes mellitus, cardiovascular diseases, hypertension, kidney diseases were excluded from the study. Healthy voluntary blood donors (HVBD) attending the blood bank of our hospital served as the control group. Thirty venous samples from the patients with AIH and 30 samples collected from HVBD which served as the control group out of which 15 males and 15 females for both the groups, were analyzed for routine liver function tests (LFT), which included total and direct bilirubin, total proteins, albumin, aspartate transaminase (AST), alanine transaminase (ALT) and alakaline phosphatase (ALP) and special investigations like SOD, CAT and MDA.

Data of the patients was collected from case files from medical records section of our institute hospital.

Venous samples received at clinical chemistry laboratory of our hospital, were allowed to clot and then centrifuged to get serum, which was then immediately analyzed for routine LFT and special investigations like SOD, CAT and MDA.

Routine LFT was analyzed by standard clinical chemistry procedures on multi-channel autoanalyzer (Beckman Coulter, Synchron CX4-PRO- Clinical System) using kits from WIPRO Ltd. Quality control samples from WIPRO Ltd. were run time-to-time on analyzer to check the accuracy and precision of these tests.

Lipid Peroxidation

The plasma MDA level was measured by the method of Satoh [7]. The assay principle is as follows: Malondialdehyde, a secondary product of lipid peroxidation reacts with thiobarbituric acid (TBA) in acidic medium to give a pink colored pigment at 97°C at pH 2-3. The pink color is extracted with butanol and the absorbance read at 530 nm.

In brief, 0.5 ml plasma was precipitated with 10% phosphotungstic acid. The mixture was centrifuged at 3000 X g for 10 min and the sediment was suspended in 4 ml distilled water. To this, 0.5 ml glacial acetic add and 0.5 ml of 0.33 TBA solutions were added, and the mixture was placed in a water bath at 97°C for 45 min. After cooling, 0.05 ml of 5M HCI was added to bring the pH of the solution to < 2 (1.6-1.7) (8). The resultant pink color was extracted with 4 ml butanol and absorbance read at 530 nm. Values were expressed as nanomoles of MDA formed per dl plasma taking the molar absorption coefficient of MDA 1.5 X 10^5 (9).

Sod Activity

The principle of SOD activity assay was based on the inhibition of nitroblue tetrazolium (NBT) reduction. Riboflavin's illumination in the presence of O_2 and electron donor like methionine generates superoxide anions and this has been used as the basis of assay of SOD. The reduction of NBT by the superoxide radicals to blue colored formazan was monitored at 550 nm. "One unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of NBT by 50% under the specified conditions." The preparation of hemolysate was done by the method of McCord and Fridovich [10]. In brief, washed erythrocytes treated with ethanol and chloroform, were centrifuged for 15 min, at 4°C, and supernatant was used for assay.

The procedure adopted was that of Beauchamp and Fridovich [11]. The reaction mixture contained, 1.9 ml of phosphate buffer (pH 7.8), 1 X 10^{-2} M methionine, 16.8 X 10^{-5} M NBT and 1.17 X 10^{-6} M riboflavin, with a similarly diluted erythrocyte hemolysate in a total volume of 3 ml. Illumination of the solution taken in 10 ml beaker was carried out in an aluminium foil lined box, with 1 15W fluorescent lamp for 10 min. Control without the enzyme source was always included. The absorbance was measured at 550 nm. The values were expressed as units/Gm Hb[12].

Cat Activity

The catalase activity of the hemolysate was determined by adopting the method of Brannan *et al*[13]. The catalase activity of the hemolysate was determined by adopting the method of Brannan *et al*[13]. The assay is based on the disappearance of H_2O_2 in the presence of the enzyme source at 30°C. In brief, the hemolysate was prepared from the lyzed RBC suspension, further diluted by phosphate buffer (pH 7.0). Here the reaction mixture containing 0.05 M buffer (pH 7.0), 1.2 mM H_2O_2 and 0.2 ml of diluted hemolysate was allowed to stand for 30 min. The reaction was stopped by the addition of 2.5 ml of the peroxide reagent containing peroxidase and chromogen system. Peroxidase reduced the H_2O_2 to produce a pink colored compound and absorbance measured at 510 nm. With each assay a suitable blank which contained no H_2O_2 and a control which contained 1 ml sodium azide, a catalase inhibitor, were included. Values were expressed as Units/ Gm Hb.

The hemoglobin content of the erythrocyte was determined according to the method employed by Rukmini *et al*[4].

Statistical Analysis

Statistical analysis was done using student's t-test. Correlation coefficient was determined between routine LFT and special investigations.

RESULTS AND DISCUSSION

The most primitive phase in an infective hepatitis is the passage of the virus into the liver parenchyma cells. In fact, this is followed by a phasic immune response of the host cell and ultimate infiltration of the infected host cell by potentially activated leucocytes. In the present study, table 1 shows a comparison between the patients and controls for the routine tests of liver function. Patients with AIH showed significantly higher levels of serum bilirubin and enzymes, namely, SGOT and SGPT as compared to that in the control group ($p < 0.001$). The noticeable point here is that the serum total bilirubin values

in the patient group ranged from 2.4 to 19.3 mg% and the direct bilirubin from 0.5 to 8.6 mg%. This wide spectrum of values is very typical of a pathological condition. Estimation of serum bilirubin is routinely carried out in patients with liver disease as a diagnostic or prognostic tool. Recent studies have reflected that bilirubin should not be considered as just a metabolic waste. It also functions *in vivo* as a potent anti-oxidant, anti-mutagen, anti-compliment and an endogenous tissue protector[15]. The increased serum bilirubin levels could be looked at as a compensatory/retaliatory phenomenon in response to cellular peroxidative changes. The same happening is true for all the enzymatic parameters except that for ALP. As far as total protein is concerned, it did not alter, although an increase in serum albumin concomitant with decrease in serum globulin were registered but rather less significant compared to bilirubin, SGOT and SGPT levels.

The table 2 summarizes the comparison between patients and controls for bilirubin, CAT, SOD and MDA. The correlation between serum bilirubin and serum MDA was significantly positive. The finding pertaining significant positive correlation between serum MDA and serum bilirubin seems to strongly support the hypothesis regarding a compensatory/retaliatory phenomenon in response to certain disease specific cellular peroxidative changes. Erythrocyte CAT and SOD activities were strongly positive correlated with MDA concentration in the patient group. This correlation was calculated taking into the consideration of the data of these parameters obtained from control group. The reduction in the erythrocyte SOD and CAT activities [16] could lead to enhancement in the release of free radicals resulting in increase in peroxidation of membrane lipids. As a consequence, there are possibilities of rupture of the lysosomal membranes, the release of lysosomal enzymes, necrosis of the cell and destruction of parenchyma tissues. All these processes culminate in an increase in serum MDA levels. Hence, increased serum MDA concomitant with decreased erythrocyte SOD and CAT activities could be used as potential markers for the free radical mediated destruction of liver parenchyma cells. These data can be strongly correlated with the data extracted from studies performed by [3]. Taken together the data of the present study with data [3], the lipid peroxidation (assessed in terms of MDA level), consequent to decreased SOD and CAT activities is followed by loss of structural integrity of plasma membranes, leading to release of membrane-associated enzymes like ADA and 5' NT into the blood circulation system in human body. This is observed as a steep enhancement in the plasma levels of these enzymes. Virus infected conditions. The assay of erythrocyte SOD, and CAT in combination with serum ADA and 5' NT can be established as strong diagnostic tools for AIH. There is a lot of controversy as regards to enzyme antioxidant levels in various virus infected conditions. There are few reports which have shown that antioxidant enzymes level increase in acute conditions which perhaps an adaptive response of these enzymes to increased production of oxygen following oxidative injury and cell damage. However in chronic condition the levels are low. Antioxidant enzymes such as SOD, catalase and glutathione peroxidase are enzymes that remove O_2 and H_20_2 from tissues. SOD activity is especially important for antioxidant defense. There are two SOD enzyme classes depending on metal content, Cu-SOD located in cytoplasm and Zn-SOD located in nucleus while manganese containing SOD is located in mitochondria, at site of oxidation and free radical production. So it is important to identify the level of particular class of SOD.

Besides, these findings provide a theoretical basis for the development of novel therapeutic strategies such as supplementation of certain specific enzymatic and/or nonenzymatic antioxidants.

Table 1: Comparative Account of Routine Liver Function Profile between Controls and patients groups.

Perimeter	References Values	Controls (n=30) mean ± SD	Patients (n=30) mean ± SD
Total Bilirubin (mg/dl)	0.2 - 1.2	0.80±0.15	7.20±4.10**
Direct Bilirubin (mg/dl)	0.0 - 0.3	0.20±0.05	2.30±0.45**
Indirect Bilirubin (mg/dl)	0.5-0.9	0.60±0.10	4.90±1.95**
Total Proteins (g/dl)	6.6-8.3	6.90±1.98	7.30±1.49*
Albumin (g/dl)	3.5-5.0	3.80±0.68	2.18±0.55*
SGOT (AST) (IU/L)	10-46	28.50±7.50	393.15±115.09**
SGPT (ALT) (IU/L)	10-49	20.10±5.60	354.35±105.18**
ALP (IU/L)	60-170	90.20±25.10	115.13±35.16*

*Not Significant
* p< 0.01
**p< 0.001
n= number of samples

Table 2: Comparison of Total Bilirubin, MDA Level, SOD and Catalase Activities in Acute Infective Hepatitis Patients and Controls.

Parameters	Control (n=30) mean ± SD	Patients (n=30) mean ± SD
Total Bilirubin (mgldl)	0.60 ± 0.15	7.20±4.10**
SOD (Units/gm Hb)	4015.16 ± 809.15	1890.31 ± 317.63*
CAT (Units/gm Hb)	5975.38 ± 1013.27	2437.54 ± 490.17*
MDA (nmol/dl)	125.35 ± 35.06	658.35 ± 136.43**

*p < 0.01
** p < 0.001
n= number of samples

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