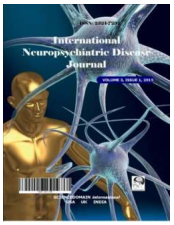

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Evaluation of Oxidative Stress in Liver Cirrhosis Patients to Early Diagnosis of Minimal Hepatic Encephalopathy

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Authors' contributions

This work was carried out in collaboration between all authors. Author KPS had carried out the study and revised the article critically for important intellectual content. Author NK contributed to the conception and design of the study and overall study supervision. Author KP was assisted in conception and design of the study, interpretation of data. Author KR was responsible for the data analysis, drafted the article, statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Minimal hepatic encephalopathy (MHE) is mildest form of the spectrum of neuropsychiatric and neurocognitive impairment in cirrhosis. Early recognition of this impairment may prevent the progression or may delay the development of the disease to overt hepatic

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encephalopathy. Although certain evidence suggest that that oxidative stress is a central component in the pathogenesis MHE, data remain controversial.
Aims: We tested the utility of glutathione peroxidase (GPx) as an oxidative stress marker of HE by comparing its levels in patients with MHE.
Study Design: The study was conducted in 60 subjects, included 30 cirrhotic patients (19 with MHE, 11 without MHE) and 30 control subjects.
Results: Cirrhosis patients with signs of MHE had decreased levels of GPx when compared to the Non-MHE and control groups. Our study provided evidence of increased oxidative stress in patients affected with MHE, as expressed by decreased serum glutathione peroxidase antioxidant. We also found a significant negative correlation between the levels of serum glutathione peroxidase antioxidant and Child-Turcotte-Pugh (CTP) score (r = -0.734, *P* = .0001).
Conclusion: Our findings revealed that the levels of the oxidative stress marker - GPx, may contribute to early diagnosis of MHE when used in conjunction with other routine markers.

Keywords: Cirrhosis; minimal hepatic encephalopathy; oxidative stress; glutathione peroxidase, child-turcotte-Pugh score.

ABBREVIATIONS

HE - Hepatic Encephalopathy; MHE - Minimal Hepatic Encephalopathy; GPx - Glutathione Peroxidase CTP - Child-Turcotte-Pugh; ROS - Reactive Oxygen Species; SODs - Superoxide Dismutases; GSH – Glutathione; HBV - Hepatitis B Virus; HCV - Hepatitis C Virus; HbsAG - HBV Surface Antigen; MMSE - Minimental state examination; NCT - Number Connection Test; EEG – Electroencephalographic.

1. INTRODUCTION

Acute and chronic liver disease leads to a serious neuropsychiatric complication known as Hepatic Encephalopathy (HE). The condition has a significant negative impact on the quality of the affected individual's life and also indicates a poor outcome [1]. Normal brain functioning of patients is altered following hepatic injury and leads to a wide spectrum of psycho-neurological symptoms that may range from somnolence and excitation to confusion and coma. These symptoms are primarily caused by brain edema, intracranial hypertension and variations in the function and morphology of brain cells [2]. Even at an early stage of HE, called Minimal Hepatic Encephalopathy [MHE], there are high rates of morbidity and strong effects in the quality of the patient's life [3]. MHE induces abnormal and progressive changes in the brain that lead to the development HE. The exact prevalence of MHE is unknown, since it is difficult to diagnose, but data indicate an incidence ranging from 30% to 84% among patients with cirrhosis [4].

Evidence shows that oxidative stress, a condition arising from an imbalance between the production of toxic reactive oxygen species including catalase, glutathione peroxidase, and superoxide dismutases and antioxidant proteins such as glutathione is implicated in the development and progression of various pathological conditions [5], such as neurodegenerative disorders [6] and acute and chronic liver diseases [7]. Several studies have shown possible involvement of oxidative stress in liver injury [8-12]. In addition, numerous animal models suggest that oxidative stress is a pathogenetic mechanism involved in HE [13-17]. Studies have also shown significant reductions in the activities of the enzymes glutathione peroxidase (GPx) and superoxide dismutase that drive cells towards apoptosis [18].

GPx is an important intracellular antioxidant that catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides to protect the organism from oxidative damage [19]. Depletion of mitochondrial and cytoplasmic glutathione increase the generation of reactive oxygen species disruption of mitochondrial transmembrane potential and mitochodrial dysfunction [20].

The burden of liver cirrhosis is increasing, especially with regard to the rise in the number of patients with alcoholic hepatitis. Hence, recognition of the complications of cirrhosis including Hepatic Encephalopathy and the need for improved management of patients affected by this disease is imperative. However, there are very few reports analyzing the link between oxidative stress and liver cirrhosis in human patients.

The aim of the present study was 1) To evaluate the levels of the oxidative stress marker glutathione peroxidase in liver cirrhosis patients with or without MHE, 2) to establish correlations between the severity of liver disease according to Child-Turcotte-Pugh scores and levels of glutathione peroxidase, 3) To compare accuracy of oxidative marker with MMSE to predict the MHE.

2. MATERIALS AND METHODS

The study was conducted in the Institute of Physiology & Experimental Medicine, Madras Medical College and Department of Hepatology, Rajiv Gandhi Government General Hospital, Chennai - 03. This study was approved by the Institution Ethical Committee, Madras Medical College and Chennai (Approval No: 20012012). All authors declare that written informed consent was obtained from the patients for publication of this report.

2.1 Selection of Subjects

2.1.1 Patients group

A total of 30 patients of both sexes in the age group between 22 and 55 diagnosed with cirrhosis without clinical evidence of encephalopathy were included in the study. All the participants were informed about the study. A written and informed consent was obtained from them.

2.1.2 Control group

Thirty patients of the same sex, and education as in the previous group served as controls. They were selected from the master health check up outpatient unit of Rajiv Gandhi Government General Hospital. The participating individuals were subjected to clinical examination and laboratory tests. A detailed clinical and medical history of the individuals was also obtained.

2.2 Diagnosis of Cirrhosis

Diagnosis of cirrhosis was based on clinical and biochemical features, and ultrasound. Patients were classified into different groups (A, B, or C) based on the severity of the illness according to Child Pugh's scoring. The Child Pugh scoring is based on biochemical features including the levels of bilirubin, albumin and prothombin values and also clinical features such as the presence of ascites and encephalopathy. The following description was used to classify the severity of cirrhosis:

1 Point was assigned if encephalopathy and ascites were absent and bilirubin levels were <2 mg/dl; albumin levels were >3.5 gm/dl; Prothrombin time was < 4 seconds),
2 points were given if encephalopathy and ascites were mild; bilirubin levels lies between 2.0 - 3.0 mg/dl; albumin levels range between 2.8 - 3.5 gm/dl; prothrombin time ranges from 4.0 - 6.0 sec and
3 points were given if encephalopathy was severe; ascites was marked; bilirubin levels > 3.0 mg/dl; albumin levels <2.8 gm/dl; prothrombin time > 6 sec.

After obtaining the cumulative points, cirrhosis was classified into: Class A (5 - 6 Points), Class B (7 - 9 Points) and Class C (10 - 15 Points).

2.3 Etiology of Cirrhosis

The etiology of cirrhosis was considered due to alcohol consumption if the intake had been more than 80 g in men and 30 g in women for more than 5 years in the absence of viral and autoimune etiologies. The diagnosis of chronic hepatitis B and C was based on HbsAG and Anti HCV viral markers. The diagnosis of autoimmune hepatitis was based on autoimmune markers such as antinuclear antibody, and smooth muscle antibody. Cryptogenic cirrhosis was diagnosed if extensive work up did not reveal any possible etiology.

2.4 Inclusion Criteria

Thirty patients with liver cirrhosis without any overt neurological signs and symptoms at the time of testing were included in our study.

2.5 Exclusion Criteria

Subjects who had a history of any neurological symptoms such as insomnia, confusion, memory loss of encephalopathy were excluded from the study. Any signs of liver cell failure such as spidernacvi, caput medusae, gynecomastia, fetor hepaticus and asterixis were noted. The following subjects were also excluded from the study:

- History of recent infection, antibiotic usage (within 6 weeks duration)

- History of recent alcohol intake (within 3 month duration)
- History of GI bleed (within 6 weeks duration)
- Usage of drugs like benzodiazepines, antipiletic drugs which affect psychometric performance
- History of shunt surgery for portal hypertension
- Patients with electrolyte imbalance
- Patients with renal impairment
- Patients with Hepatocellular carcinoma
- Patients with medical disorders such as diabetes mellitus, Hypertension
- Patients with Any psychiatric illness
- Patients with Hearing defects
- Patients with Visual defects
- Illiteracy and inability to perform psychometric tests

2.6 Mini-mental State Examination

The Folstein Mini-Mental state examination is the most widely recognized and used bed side screening measure for global cognitive functioning. It consists of a total of 30 points. 5 points were assigned for time orientation, 5 points for place orientation, 5 points for attention, 3 points for registration of 3 items, 3 points for recall of 3 items after 5 minutes, 2 points for naming objects, 1 point for repeating phrase, 1 point for printed command, 1 point for writing a sentence and 1 point for copying a diagram.

2.7 Number Connection Test

It is a test of visuospatial orientation and psychomotor speed. The subject was given a sheet consisting of 25 numbered circles randomly spread over the sheet and asked to connect the numbers from 1 to 25 as quickly as possible. The time taken for completion of the task including the time taken for correction of error was also noted. A Low score indicated better performance.

2.8 Diagnosis of MHE

MHE was diagnosed by spectral electroencephalographic (EEG) features and performance in age-and education-adjusted validated paper and pencil psychometric tests (Number connection test). Cirrhosis patients were considered to have MHE if at least one measure (psychometric tests and/or EEG) was abnormal. On the basis of this criteria, 19 patients were classified as having MHE.

2.9 Glutathione Peroxidase (GPx) Estimation

The Fortress kit was used for quantitative measurement of GPx. The principle behind the test is the enzyme GPx catalyses oxidation of glutathione by cumene hydroperoxide. Oxidized glutatithione is further converted to reduced glutathione by glutathione reductase and NADPH. In this reaction NADPH is converted to NADP simultaneously. The following protocol was used according to the manufacturer's procedure: Heparinised whole blood was mixed with drabkin's reagent in the radio of 1:10 to quench any peroxidase activity; 0.5 ml of diluted sample was added to 24 well plates (0.5 ml of distilled water was taken while calculating reagent blank); 2.5 ml of kit reagent and 0.1 ml of cumene (prepared by diluting cumene hydroperoxide in the kit) were added to the same well and were mixed at room temperature. Results were read at 340 nm wavelength using an ELISA plate reader. The following calculation was done for blank and sample to obtain optical density value.

- Reading for the sample alone was taken after 1 minute.
- One more reading for the sample was taken after 1 minute.
- Reading was subtracted from the blank value.
- Calculation was done by multiplying the OD value by 8412 (a standardized value given by the kit manufacturer).
- Result was expressed as IU/L.

2.10 Statistical Analysis

Statistical analysis was done by using statistical packages for social sciences 20 (SPSS 20) software. The results were presented as mean ± sd, median (interquartile range) and proportion. Student's T test was used to compare continuous variables between two groups. The correlation between two variables was found using Chi-square for a comparison of categorical variables and a Mann-Whitney test for unpaired continuous variable data. A value of $p<0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Characteristics of Study and Control Subjects

The distributions of controls and cirrhotic patients were uniform according to age, gender and

educational status (Table 1), consisting of 30 cirrhotic patients including 19 with MHE and 11 without MHE with ages ranging from 22 - 55 years. There were 30 control subjects including 10 females and 20 males with ages ranging from 22 - 55 years. The mean age was 38.9±9.2 in controls and the mean age of cirrhotic subjects was 39.6±8.7. The duration of illness in cirrhotic patients varied from 1.5 to 6 years. Regarding their literacy status, subjects in both groups have completed at least primary school education and were able to perform number connection tests.

Considering the socio-economic profiles of the study population, most female subjects were housewives; some were employed in hostels, departmental stores, industries, and a few were involved in agricultural work. Some of the males worked in tea shops or shoe companies whereas others were involved in driving, security jobs, tailoring and agricultural work.

Cirrhotic subjects were classified according to etiology. Sixteen had alcohol induced cirrhosis and the remaining 14 had a non-alcohol related cirrhosis due to Hepatitis B virus (n - 8), hepatitis C virus (n - 2) and cryptogenic cirrhosis (n - 4). Based on the severity of illness, it was found that 5 out of 30 patients were assigned to Child-Pugh's class A with a score of 5 - 6 points and the other 25 patients to the Child Pugh class B with a score of 7 - 9 points (Table 2).

3.2 Glutathione peroxidase

Significantly lowered levels of glutathione peroxidase were measured in patients of the cirrhosis population when compared to controls. The glutathione peroxidase value in patients with cirrhosis was lower than in control subjects. We also found a considerable decrease in level of GPx in MHE (median - 1009.4), non-MHE group (median - 1640.3) as compared to the controls (median - 3575.1) (Fig. 1). In addition, a

Table 1. Comparison of characteristics between cirrhosis subjects and control subjects

Variables	Cirrhosis subjects	Controls subjects	p Value
Age (years) [Mean±SD]	39.60±8.685	38.87±9.187	0.752
Male gender	19 (63.3%)	20 (66.7%)	0.787
Bilirubin [Mean±SD]	1.207±0.4593	0.383±0.1487	0.0001
Glutathione peroxidase	1198.71 (956.865 - 1545.7050)	3575.1 (3228.1050 - 3827.46)	0.0001
MMSE [Mean±SD]	29.9±0.305	29.9±0.305	1.0

Table 2. Comparison of characteristics between cirrhosis subjects with MHE and non-MHE subjects

Variables	Cirrhosis with MHE subjects (n=19)	Cirrhosis with non-MHE subjects (n=11)	*P* Value
Age (years) [Mean±SD]	39.00±9.690	40.64±6.918	0.05
Male gender	13 (68.4%)	6 (54.5%)	0.447
Duration of cirrhotic disease [Mean±SD]	2.91±1.868	2.75±1.226	0.462
Causes of cirrhosis			
Alcoholism	10 (52.6%)	6 (54.5%)	0.521
Hepatitis B	4 (21.1%)	4 (36.4%)	
Hepatitis C	2 (10.5%)	-	
Cryptogenic	3 (15.8%)	1 (9.1%)	
Severity of illness			
Child Pugh A		5 (45.5%)	0.01
Child Pugh B	19 (100%)	6 (54.5%)	
CTP Score	8 (7 - 8)	6 (6 - 7)	0.001
Glutathione peroxidase	1009.44 (757.08 - 1261.8)	1640.34 (1430.04 - 1724.46)	0.0001
MMSE [Mean±SD]	29.84±0.375	30.00±0.0	0.002
Bilirubin [Mean±SD]	1.295±0.54	1.055±0.2162	0.001

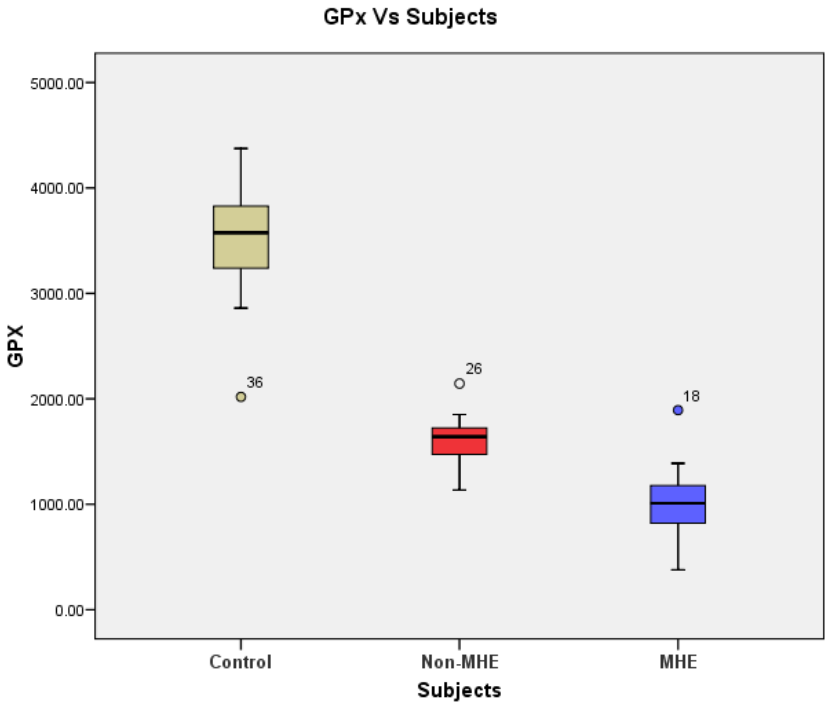

Fig. 1. Glutathione peroxidase levels in Control, MHE, non-MHE cirrhosis groups

statistical significance was found between MHE group, and control group (*P* = .0001) and significant differences between MHE vs non-MHE (*P* = .0001) in non-parametric Mann-Whitney U test analysis.

3.3 Correlation Coefficient Analysis of GPx vs Severity of Disease

Significant negative correlations was observed between the oxidative stress marker (GPx) and Child-Turcotte-Pugh scores (r= -0.734, *P* = .0001).

3.4 Minimental State Examination

There were no significant differences in mini-mental state examination scores in both the control and cirrhotic subjects. The average score in the controls was 29.9±0.3 and the mean score among cirrhotic subjects was 29.9±0.3 (*P* = 1.0).

3.5 Discussion

Hepatic encephalopathy is a serious central nervous system disease caused by liver failure. A rapid worsening of liver function results in brain damage commonly associated with excitation, confusion and coma. The development of HE is considered a sign of a poor prognosis and patients should receive therapy for an indefinite period of time or until they undergo liver transplantation. Therefore hepatic encephalopathy must be identified to initiate treatment. Hence the primary objective of our study was to estimate the oxidative stress marker i.e glutathione peroxidase-GPx as a marker of early detection HE in our region.

The lower level of the antioxidant enzyme glutathione peroxidase in patients with cirrhosis indicated a severe oxidative stress explained by its utilization in scavenging the free radicals. Excessive generation of free radicals leads to inactivation of enzymes [21]. Free radicals such as reactive oxygen species and lipid peroxides are induced by alcohol or various viral infections. According to Dart RC et al., alcohol consumption produces metabolic disturbances in the liver with the formation of reactive oxygen species leading to hepatocyte necrosis and apoptosis [22]. Another study carried out by Natarajan SK et al., have also shown evidence of oxidative damage in liver cirrhosis [23]. Hence, we interpret the decreased level of GPx enzyme activity as a result of the impaired GPx activity in cirrhosis subjects.

In contrast to our study, the report by Geetha et al., showed significantly higher levels of glutathione peroxidase in red cells of patients with cirrhosis as compared to controls [24]. Oxidative stress was found to be higher in cirrhosis with bleeding complications. Similarly, a study by Sowjanya et al. [25], also showed higher levels of peroxidants and lower levels of antioxidant enzymes such as catalase and superoxide dismutase. However, the level of glutathione peroxidase was higher in cirrhotic patients when compared to controls. They estimated the GPx levels as suggested by Paglia et al. and the higher level of glutathione peroxidase in both the above mentioned studies was attributed to counter regulation by oxidative stress.

The present study showed decreased level of GPx suggesting that cirrhosis leads to oxidative stress exhibition via the reduced level of GPx. Subjects with MHE had even lower levels of the GPx when compared to the non-MHE. This suggested that the MHE condition was highly related to oxidative stress.

Sumit bhandari et al. [26] also studied antioxidant and peroxidant levels in cirrhosis with respect to functional compromise of liver, as determined by Child Pugh scoring. The level of antioxidant enzymes such as superoxide dismutase, catalase were significantly lower in cirrhotics of Child Pugh class C, compared to classes A and B, thus suggesting that increased liver damage is associated with increase in oxidative stress.

3.6 MMSE scoring and diagnosing of MHE

The MMSE scoring was not different between cirrhotic and control groups, suggesting that none of the patients suffered from gross cognitive impairment. A score lower than 24 was considered the cut off point for the diagnosis of cognitive impairment. Normal finding in MMSE rules out the presence of clinically overt encephalopathy. The present study is similar to that by Juan Quero et al. [27] who showed normal mental status assessment by MMSE despite the abnormalities in other psychometric tests. Likewise the study by Wright CB et al. [28] in 1996 stated that MMSE has a limited ability to detect subclinical cognitive impairment among Community-based cohort population.

At present, there is no consensus on routine testing for MHE. Recent recommendations are mostly focused on testing and treating patients who exhibit symptoms like poor memory, lack of concentration, difficulties in performing everyday activities, or for patients who are at an increased professional risk of accidents (i.e. drivers). In case of identifying MHE, there is a definite need to prevent development of HE. The importance of its early diagnosis also lies in the improvement of the quality of life and work capacity of these patients, thereby helping to alleviate the consequences of this minor but still crucial cognitive and neurological disorder.

4. CONCLUSION

The diagnostic methods suggested in the present study allow the identifycation of functional disturbances before the onset of symptoms. Behavioral changes, avoidance of predisposing factors or timely treatment can help postpone or even avoid clinical forms of decompensated liver cirrhosis. There is a definite role of oxidative stress in patients with liver cirrhosis, as evidenced by the reduced glutathione peroxidase level. However, the subtle cognitive deficit was not evident by MMSE. The outcome of this study helps to throw light on the role of glutathione peroxidase in early diagnosis of MHE along with other criteria. This would help in the early implementation of treatment so that progression to overt encephalopathy could be prevented.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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