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3-Nitro-Tyrosine as a Biomarker of Minimal Hepatic Encephalopathy in Patients with Liver Cirrhosis

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ABSTRACT

Background: hepatic encephalopathy (HE) is a common complication in patient with liver cirrhosis. It comprises of a broad spectrum of neuropsychiatric abnormalities of varying severity, and affected patients usually suffer from psychomotor, cognitive, emotional, behavioural, and motor coordination dysfunctions. Patients with minimal HE (MHE), a subclinical form of HE, usually have a normal mental and neurological status upon routine clinical examination. The subtle deficits in patients with MHE can only be elicited by specialized neuropsychological tests. **Aim of the Work:** the aim of this study was to evaluate the role of 3-Nitro-Tyrosine as a biomarker of Minimal Hepatic Encephalopathy in patients with liver cirrhosis. **Patients and Methods:** our conducted study was a prospective case control study carried on 60 adult patients and 30 age matched controls. All were recruited from Internal Medicine and Hepatology and Gastroenterology Department at Ain Shams University Hospitals in the period between September 2016 and June 2018. All patients enrolled in the study were subjected to detailed history taking, full physical examination, laboratory investigations, psychometric tests for detection of MHE using specially digit symbol test (DST), Trail making test A (TMT A), Trail making test B (TMT B), serial dotting test (SDT) and 3-Nitro-Tyrosine level (3NT). **Results:** our study found that the serum levels of 3-nitro-tyrosine are a good predictor of the presence of MHE in patients with liver cirrhosis, with good sensitivity (90%) and specificity (93.33%) and positive and negative predictive values were 93.1% and 90.3% respectively at a cutoff of 14.8 ng. **Conclusion:** determination of 3-nitro-tyrosine in serum is easy and is not time consuming. It only requires taking a serum sample from the patient and determining 3-nitro-tyrosine concentration. This procedure can be therefore easily added to the routine clinical determinations in patients with liver cirrhosis. This would also allow extending the diagnosis of MHE to most clinical settings, helping to identify patients with MHE.

Keywords: 3-Nitro-Tyrosine, Hepatic Encephalopathy, Liver Cirrhosis

INTRODUCTION

Liver disease is a major cause of mortality and morbidity worldwide. In most cases, liver-related mortality results from complications of chronic liver disease (CLD) including advanced cirrhosis and hepatocellular carcinoma (HCC) [(1)]. Chronic Liver Disease and complications of cirrhosis including ascites, bleeding tendency, Hepatocellular Carcinoma, minimal and overt hepatic encephalopathy also are associated with severe impairments in health-related quality of life [(2)]. The burden of liver disease in Egypt is exceptionally high, maintaining the highest prevalence of hepatitis C virus (HCV) worldwide [(3)]. Hepatic encephalopathy (HE) is a frequent complication and one of the most debilitating manifestations of liver disease, severely affecting the lives of patients and their caregivers. Furthermore, cognitive impairment associated with cirrhosis results in utilization of more health care resources in adults than other manifestations of liver disease. Progress in the area has been hindered by the complex pathogenesis that is not yet fully elucidated [(4)]. Apart from such biological factors, there remains the larger obstacle that there are no universally accepted standards for the definition, diagnosis, classification, or treatment of HE, mostly as a result of insufficient clinical studies and standardized definitions. Clinical management tends to be dependent on local standards and personal views. This is an unfavorable situation for patients and contrasts with the severity of the condition and the high level of standardization in other complications of cirrhosis [(4)]. Between 30 and 50 % of the patients with liver cirrhosis who do not show evident symptoms of clinical hepatic encephalopathy (HE) have minimal HE (MHE). MHE cannot be detected in routine analysis but can be unveiled using psychometric tests or neurophysiological assessment [(4,5)]. MHE has a negative impact on daily life activities and working capacity, affects health-related quality of life (HR-QoL) impairs fitness to drive [(4)]. Currently, the ‘‘gold standard’’ for diagnosis of MHE is the psychometric hepatic encephalopathy score (PHES), a battery of five psychometric tests. However, PHES is time consuming and needs adjusting for age and educational level. As a consequence, MHE is not routinely diagnosed in most clinical settings because of lack of simple procedures, and most patients with MHE remain undiagnosed and untreated. Hence, there is a need for a simple diagnostic test that can be performed routinely in the laboratory to detect MHE in patients with liver cirrhosis [(5)]. It would be very useful in clinical practice to have some

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peripheral biomarker that could be measured in blood samples and reflect the presence of MHE in cirrhotic patients. We have been looking for such possible peripheral indicator during the last few years. We showed that the presence of MHE correlates with increased activation of soluble guanylate cyclase by nitric oxide in freshly isolated lymphocytes, and that increased serum levels of pro inflammatory cytokines, interleukin (IL)-6 and IL-18 also correlate with MHE and would be useful to detect MHE [(4)].

AIM OF THE WORK

The aim of this study was to evaluate the role of 3-Nitro-Tyrosine as a biomarker of Minimal Hepatic Encephalopathy in patients with liver cirrhosis.

PATIENTS AND METHODS

This is a prospective study at Internal Medicine and Hepatology and Gastroenterology Department at Ain Shams University Hospitals in the period between September 2016 and June 2018. **The study was approved by the Ethics Board of Ain Shams University and an informed written consent was taken from each participant in the study.** It include sixty Egyptian patients selected from Internal Medicine and Hepatology outpatient clinics and inpatient wards at Ain Shams University Hospitals and classified into 2 groups according to the following inclusion criteria: Group I: 30 cirrhotic patients with MHE, Group II: 30 cirrhotic patients without MHE. ***Control group:** 30 healthy subjects. ***All the following was done to the patients as well as controls: Biochemical studies***: Complete blood picture (CBC), liver function tests: alanine transaminase (ALT), aspartate transaminase (AST), Total and Direct bilirubin, international normalised ratio (INR), Prothrombin time (PT), serum albumin, Kidney function tests and full electrolytes: Blood urea nitrogen (BUN), creatinine, sodium, potassium, Magnesium, Calcium, Phosphorus. ***3-Nitrotyrosine level***: 3-Nitrotyrosine ELISA Kit is a competitive enzyme-linked immunosorbent assay for the quantitative measurement of 3-Nitrotyrosine in serum, plasma, cell culture supernatants, tissue homogenates and other biological fluids. ***Principle of the Assay:*** This ELISA kit uses Competitive-ELISA as the method. The microtiter plate provided in this kit has been pre-coated with 3-NT. During the reaction 3-NT in the sample or standard competes with a fixed amount of 3-NT on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to 3-NT. Excess conjugate and unbound sample or standard are washed from the plate, and HRP-Streptavidin (SABC) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of 3-NT in the samples is then determined by comparing the OD of the samples to the standard curve. ***Normal range****: 0.5 – 10 ng/ml.* ***Psychiatric assessment as well as psychometric tests***: The neuropsychological test battery applied in this study was based on the recent data on sensitivity and specificity of psychometric tests in hepatic encephalopathy [(6)]**. Exclusion criteria:** Overt HE (Grade 1,2,3 and 4 in West Heaven classification [(7)]**).** Uncontrolled diabetes with high levels of glycosylated hemoglobin, renal dysfunction, hyponatremia, concomitant neurological disease, severe cardiovascular disease, evidence of overt infection on clinical assessment, transjugular intrahepatic portosystemic shunt, no history of spontaneous bacterial peritonitis. **Statistical analysis:** Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation and Student t-test [Unpaired], Paired t-test, Linear Correlation Coefficient [r] Chi-square and ROC curve by SPSSV17. Unpaired Student t-test was used to compare between tow groups in quantitative data. Unpaired Student t-test was used to compare between related sample. Chi-square the hypothesis that the row and column variables are independent, without indicating strength or direction of the relationship. Pearson chi-square and likelihood-ratio chi-square. Fisher's exact test and Yates' corrected chi-square are computed for 2x2 tables. Linear Correlation coefficient was used for detection of correlation between two quantitative variables in one group.

RESULTS

Table (1): Comparison between patients and control as regards sex

Sex		Groups		Total
		Patients	Controls	
Male	N	47	19	66
	%	78.3 %	63.3 %	73.3 %
Female	N	13	11	24
	%	21.7 %	36.7 %	26.7 %
Total	N	60	30	90
	%	100.0%	100.0%	100.0%
Chi-square	Test value	2.30		
	P-value	0.129		

Table (2): Comparison between patients and control as regards Age

		Groups	
		Patients	**Controls**
Age	Mean ± SD	57.42 ± 7.36	27.90 ± 8.29
	Range	41 – 71	20 – 55
Chi-square	**Test value**	-17.190	
	P-value	<0.001*	

Table (3): Showing child score of patients (with MHE and no MHE)

Patients	**Child score**			**Paired T-test**	
	Mean	**±**	**SD**	**T**	**P-value**
with MHE	11.97	±	1.99	173.278	<0.001*
No MHE	6.70	±	0.92		

Table (4): Showing MELD score of patients (with MHE and no MHE)

Patients	**MELD**			**Paired T-test**	
	Mean	**±**	**SD**	**T**	**P-value**
with MHE	17.23	±	2.97	163.886	0.003*
No MHE	8.57	±	2.22		

Table (5): Showing serum 3-Nitro-Tyrosine level in patients (with MHE and no MHE) and control

		No MHE	**MHT**	**Control group**	**Test value•**	**P-value**
3-Nitro-Tyrosine level	Mean±SD	6.16 ±0.99	50.19±3.09	3.82±0.67	5607.689	<0.001*
	Range	5-7.5	44.5-55.1	3.1-5.1		

Table (6): Relation between total score of the four psychometric tests (PHES) and other parameters

All Patient	**TOTAL of 4 tests**	
	r	**P-value**
3-Nitro-Tyrosine	-0.787**	< 0.001
Bilirubin	-0.550**	< 0.001
INR	-0.355**	0.005
AST	-0.031	0.811
ALT	-0.318*	0.013
Albumin	0.592**	< 0.001
Sodium	0.138	0.294
Potassium	0.153	0.243
Creatinine	-0.120	0.362
Child score	-0.601**	< 0.001
MELD	-0.542**	< 0.001

Table (7): Relation between 3-Nitro-Tyrosine and other parameters.

All Patient	**3NT**	
	r	**P-value**
TOTAL score of 4 tests	-0.787**	< 0.001
Bilirubin	0.649**	< 0.001
INR	0.449**	< 0.001
AST	0.050	0.705
ALT	0.359**	0.005
Albumin	-0.671**	< 0.001
Sodium	-0.090	0.492
Potassium	-0.231	0.076
Creatinine	0.153	0.243
Child score	0.814**	< 0.001
MELD	0.799**	< 0.001
TMT A per sec	0.619**	< 0.001
TMT B per sec	0.782**	< 0.001
DST points	-0.918**	< 0.001
SDT per sec	0.806**	< 0.001

DISCUSSION

Liver cirrhosis is associated with several serious complications, including ascites, spontaneous bacterial peritonitis, variceal bleeding, and hepatic encephalopathy (HE) [6]. HE is frequently associated with a wide range of neuropsychiatric abnormalities in liver cirrhosis, and has been classified as a continuum from minimal HE (MHE) to different grades of overt HE. Minimal hepatic encephalopathy (MHE) is the mildest form of the spectrum of neurocognitive impairment in cirrhosis [7]. The prevalence of Overt HE is 16%–21% in those with decompensated cirrhosis while Minimal HE (MHE) or covert HE (CHE) occurs in 20%–80% of patients with cirrhosis [8]. MHE is detectable only by specialized neurocognitive testing. It is a clinically significant disorder which impairs daily functioning, driving performance, work capability and learning ability. It also predisposes to the development of overt hepatic encephalopathy and increased mortality. This results in impaired quality of life for the patient as well as significant social and economic burden for health providers and care givers [9]. It is believed that cerebral edema is the common pathogenic mechanism for cognitive impairment in MHE and overt HE [7]. There are several methods of diagnosing MHE, including comprehensive neuropsychological examinations, standard psychometric batteries, neuro physiological testing, and computerized testing. However, there are no

current guidelines for the standardized diagnosis of MHE, there is no gold standard for diagnosis of MHE **(10).** The psychometric hepatic encephalopathy score (PHES) battery is a reliable tool to detect neuropsychiatric dysfunction. It assesses multiple cognitive domains in patients with cirrhosis, including visual perception, construction, visual/spatial orientation, motor speed and accuracy, concentration, and attention **(11).** The PHES initially comprised of seven tests but the desire for a shorter battery and the poor sensitivity of some of the tests led to the introduction of a revised battery called the Portosystemic Encephalopathy (PSE) Syndrome Test, which includes the line tracing test (LTT), digit symbol test (DST) and number connection test A and B (NCT A and B) and serial dotting test (SDT). The sensitivity and specificity of the PHES compared with the standard method of determining HE grade were 96% and 100%, respectively **(12).** A score of below or equal to -4 is diagnostic for minimal hepatic encephalopathy **(11).** Three of the four tests, NCT-A, NCT-B and DST, have been commonly used for the detection of MHE. The result of a single test was regarded to be abnormal if the result was beyond the 2 SD ranges of the control norms. In some studies, MHE was diagnosed when two tests were abnormal **(13).** Compared to PHES, NCT-A and DST were able to diagnose MHE with a sensitivity of 76.9% and a specificity of 96.3% **(14).** The following neuropsychological test battery consisting of TMT A, TMT B, SDT and DST was done to control group as well as patients. These are the same tests used to diagnose MHE by many studies as Ji-Yao ***et al.*** **(10)** and Tsai ***et al.*** **(15).** Blood ammonia level is not measured in this study as it's proved that its blood level doesn't not correlate with psychometric tests score and the occurrence of MHE **(16)**. This agree with Ji-Yao ***et al.*** (10) ***and*** Tsai ***et al.*** (15) that also did not use ammonia level and disagree with Lockwood **(17),** Shawcross ***et al.*** (18)**,** that use it. Other studies only use it to monitor the level as part of the investigations done but not a proof for diagnosis or exclusion of MHE **(7).** The total score of the following four tests (TMT A, B, SDT and DST) is calculated with a cut off point of -4. Any patient with a score of below or equal to -4 is defined as having MHE. This cut off value is used by studies to diagnose MHE as Ji-Yao ***et al.*** (10)**,** Tsai ***et al.*** (15) and Zhang ***et al.*** **(19)**. This is the first peripheral biomarker described up to now for diagnosis of MHE in patients with liver cirrhosis. Determination of 3-nitro-tyrosine in serum is easy and is not time consuming and can be therefore easily added to the routine clinical determinations in patients with liver cirrhosis. This would allow extending the diagnosis of MHE to most clinical settings, helping to identify patients with MHE.

CONCLUSION

HES is time consuming and needs adjusting for age and educational level. As a consequence, MHE is not routinely diagnosed in most clinical consequence because of lack of simple procedures, and most patients with MHE remain undiagnosed and untreated. Determination of 3-nitro-tyrosine in serum is easy and is not time consuming. This would also allow extending the diagnosis of MHE to most clinical settings, helping to identify patients with MHE. Serum levels of 3-nitro-tyrosine are a good predictor of the presence of MHE in patients with liver cirrhosis, with good sensitivity (90%) and specificity (93.33%) and positive and negative predictive values were 93.1% and 90.3% respectively at a cutoff of 14 ng.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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