Original Article

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Biochemistry Section

Comparison of TNF-α Levels in Alcoholic Liver Disease Patients with and without Hepatitis: A Cross-sectional Study in Coastal Karnataka, India

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ABSTRACT

Introduction: Alcohol-induced liver injury triggers inflammatory reactions through pro-inflammatory cytokine Tumour Necrosis Factor-α (TNF-α). The estimation of TNF-α would provide valuable information on the degree of activation of immune system and inflammation in the liver cells, which is crucial in the diagnosis as well as prognosis of alcoholic hepatitis.

Aim: To compare TNF-α levels in Alcoholic Liver Disease (ALD) patients with or without hepatitis.

Materials and Methods: This cross-sectional study was conducted at Yenepoya Medical College Hospital, Mangaluru, Karnataka, India from October 2021 to June 2022. The study included 52 patients diagnosed with Alcoholic Liver Disease (ALD), aged between 45-75 years which were equally divided into two groups (n=26 each): ALD with hepatitis and ALD without hepatitis. The diagnosis of hepatitis was made based on the De Ritis ratio (>2) along with a history of alcohol consumption. The Liver Function Test (LFT) was estimated using the automated system. Child-Pugh (CP) score was assessed by scoring serum albumin, total bilirubin, Prothrombin Time (PT), ascites, and grades of hepatic encephalopathy. The TNF-α was estimated by the Enzyme Linked Immuno Absorbent Assay (ELISA). The LFT, CP score, and TNF-α levels were compared between ALD patients with or without hepatitis using Independent sample t-test.

Results: All the study participants were male, with the median age of those with alcoholic hepatitis being 50.23 years. Around 65% of them had severe liver dysfunction with higher CP scores (p-value=0.001). Platelet count was significantly lower in alcoholic hepatitis, resulting in prolonged PT. Patients with alcoholic hepatitis had significantly high levels of bilirubin (p-value=0.006), aspartate transaminase (p-value=0.001), alkaline phosphatase (p-value=0.001), and TNF-α (p-value=0.005).

Conclusion: The study concluded that the patients of ALD have elevation of TNF-α levels, when they progress to or have episodes of active hepatitis.

Keywords: Alcohol induced liver injury, Child-pugh score, De Ritis ratio, Inflammatory cytokines, Tumour necrosis factor-α

INTRODUCTION

The ALD is a spectrum of disease which includes combination of both clinical and histological features, such as fatty liver (steatosis), alcoholic hepatitis, and cirrhosis. The pattern ranges from mild hepatosteatosis, which is reversible and asymptomatic, to further progression into fibrosis, and may end up in hepatocellular carcinoma [1]. The most common causes of cirrhosis worldwide are related to viral hepatitis, followed by alcohol [1,2]. Global prevalence of alcohol-associated cirrhosis has been estimated at 23.6 million for compensated cirrhosis and 2.46 million individuals for decompensated cirrhosis, respectively [3]. The overall pooled estimate of alcoholic cirrhosis in adults in India is 43.2%, with alcohol remains major cause for cirrhosis in all zones [4].

Early detection and intervention are essential for managing ALD and prevent its progression to advanced liver disease. The thorough medical history about quantity and duration of alcohol consumption, clinical feature of hepatomegaly along with abnormal LFTs help in considering the diagnosis of ALD. ALD patients with hepatitis and without hepatitis are differentiated by the estimation of serum levels of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT). There is a need to identify the marker which not only can differentiate ALD with or without hepatitis but also be used as a target in management as well. In advanced and severe cases of ALD, clinical and laboratory parameters can help determine the prognosis and the best therapeutic approach [5,6].

There are various scoring methods available, such as model for End-Stage Liver Disease (MELD) score [7], Glasgow Alcoholic Hepatitis Score [8], Age, Bilirubin, International Normalised Ratio (INR), Creatinine (ABIC) score [9], and CP score [10], to evaluate the severity and prognosis of patients with cirrhosis, including those with ALD. CP score is easy to use and is mainly used to assess the liver disease patient. However, there are few challenges in using this score in evaluating severity of liver disease, as CP score include subjective assessment like grading of ascites and encephalopathy [10]. Cytokines are appropriate markers in ALD, as hepatic cell injury by chronic alcohol consumption triggers inflammatory reactions mediated by inflammatory cytokines like TNF-α. It is one of the earliest events in many types of liver injury [11].

Most previous studies have focused on correlation of TNF-α among ALD patients as a whole or differentiated them as alcoholic or non alcoholic liver disease patients [12,13]. However, the current study differentiates the role of TNF-α in ALD with or without hepatitis and correlating it with severity score (CP score) would give more insight about the management of ALD patients. Hence, this study aimed to explore the role of TNF-α in ALD patients, with or without hepatitis.

MATERIALS AND METHODS

This cross-sectional study was conducted at Yenepoya Medical College Hospital, Mangaluru, Karnataka, India from October 2021 to June 2022, after obtaining ethical clearance ((YEC2/924). The study included 52 patients diagnosed with Alcoholic Liver Disease (ALD),

with IEC approval number (YEC2/924). The study adhered to the ethical guidelines established by the 1964 Helsinki Declaration and its subsequent modifications. The study participants were included after obtaining written informed consent.

Inclusion criteria: Patients aged between 45 and 75 years with established ALD were included in the study after obtaining informed consent. A total of 52 patients were divided into two groups: 26 patients of ALD with hepatitis and 26 patients of ALD without hepatitis. The diagnosis of ALD with hepatitis was made using the De Ritis ratio (AST/ALT ratio). Patients with a De Ritis ratio >2 were classified as having ALD with hepatitis, while those with a De Ritis ratio ≤2 were classified as having ALD without hepatitis [14].

Exclusion criteria: Patients with Non Alcoholic Fatty Liver Disease (NAFLD) were excluded from the study.

Sample size calculation: The sample size was calculated using G*Power software, with a power of 80%, a significance level of 5% (α=0.05), and a standard effect size of 0.8. An effect size of 0.8 was selected based on the standard effect size for t-test with large effect [15]. The sample size was calculated as 26 in each group of ALD patients, with or without hepatitis.

Study Procedure

Five millilitre blood was collected from each study participants. After storing 500 µL of serum sample for the analysis of TNF-α at -20°C, rest of serum samples were used for analysis of other biochemical parameters. Serum total bilirubin (0-1.3 mg/dL), liver enzymes such as AST (0-35 U/L), ALT (0-45 U/L), and Alkaline Phosphatase (ALP) (60-170 U/L), total protein (6.3-8.2 g/dL), and albumin levels (3.2-4.4 g/dL) were estimated by VITROS microslide method using ORTHO VITROS 5600 Integrated system [16]. A history of alcohol dependence was taken from patients. Details of Red Blood Cell (RBC) count (4.5-5.5 million/cumm), White Blood Cell (WBC) count (4-11×10³ µL), platelet count (150-450 ×10³ µL), and Prothrombin Time (PT):11.9-14.8 secs [17], as well as information on ascites and grades of hepatic encephalopathy, were taken from medical records of patients. Estimation of TNF-α was done by ELISA technique using commercially available kit with an assay range of 7.8-500 ng/mL [18].

Child-Pugh score assessment: The Child-Pugh (CP) score was calculated by evaluating five clinical parameters of liver disease: serum albumin levels, prothrombin time, total bilirubin, presence of ascites, and severity of hepatic encephalopathy. Each parameter was scored from 1 to 3 points, with 3 indicating the most severe manifestation. Based on the cumulative score, patients were categorised into three prognostic categories:

Class A (5-6 points): Well-compensated disease

Class B (7-9 points): Significant functional compromise

Class C (10-15 points): Decompensated disease

This scoring system serves as a valuable tool for predicting mortality risk in patients with alcoholic liver disease [10].

STATISTICAL ANALYSIS

Descriptive statistics was done to provide the demographic and clinical characteristics of the study participants. The normality assumption was verified using Shapiro-Wilk test. The independent sample t-test was used to compare the cases and controls for parametric data. Results are expressed as mean±standard deviation. Mann-Whitney U test was used for non parametric data. Spearman correlation was used to find the relation between CP score and AST, ALT, ALP, bilirubin, and TNF-α. A p-value of <0.05 was considered statistically significant. All the statistical analysis were done using IBM Statistical Package for Social Sciences (SPSS) Statistics software (version 23.0).

RESULTS

This study was carried out to compare serum levels of TNF-α in patients with ALD with hepatitis and without hepatitis. A total of 26 patients in each group were recruited in the study and the levels of serum TNF-α was measured in serum samples and CP score was also calculated.

All the study participants were male. The mean age of patients of ALD with or without hepatitis was 51±5 years and 52±5 years, respectively. The LFT parameters, such as total bilirubin, total protein, albumin, AST, ALT, and ALP, were compared between cases and controls. The levels of total bilirubin (p-value=0.006), AST (p-value=0.001), and ALP (p-value=0.001) were significantly higher in patients of ALD with hepatitis than patients without hepatitis. Since this is alcoholic hepatitis, the level of AST is significantly higher than controls. ALT level was lower in patient with hepatitis. The level of albumin was significantly lower in alcoholic hepatitis patients (p-value=0.001). Total protein level was lower in hepatitis patients (6.85 gm/dL) compared to patients without hepatitis (7.25 gm/dL), but the decrease was not statistically significant [Table/Fig-1].

S. No.	Variables	Total study population (N=52)	With hepatitis (n=26)	Without hepatitis (n=26)	p-value
1	Total bilirubin (mg/dL)	2.25 (0.88-4.03)	3.25 (1.53-5.5)	1.2 (0.68-3.15)	0.006*
2	AST (IU/L)	68 (41.5-89.8)	85 (68-136.8)	45.5 (37.5-66.25)	0.001*
3	ALT (IU/L)	31.5 (22-44)	30.5 (19.8-38)	32 (22.75-60)	0.245
4	ALP (IU/L)	136 (94.8-178)	174 (136.25-227.5)	103 (90-130)	0.001*
5	Total protein (gm/dL)	7 (6.47-7.60)	6.85 (6.2-7.45)	7.25 (6.73-7.73)	0.164
6	Albumin (gm/dL)	2.9 (2.5-3.23)	2.5 (2.2-2.9)	3 (2.9-3.93)	0.001*
7	Child-Pugh (CP) score	9 (7-11)	10.5 (8.5-12)	7.5 (6-9)	0.001*
8	$TNF-α (pg/mL)	289.96±162.95	352.52±160.70	227.40±142.13	0.005*

[Table/Fig-1]: Biochemical parameters of total study population=52.
Data presented as median (IQR) for non parametric variables (Mann-Whitney U test) and mean±SD (TNF-α) for parametric variables (Independent sample t-test)
*p<0.05 considered statistically significant. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase

Whereas CP score also showed significant difference between two groups (p-value=0.001), The ALD patients with hepatitis patients had high CP score compared to another group. The levels of TNF-α were higher (352.52±160.70 pg/mL) in alcoholic hepatitis patients than ALD patients without hepatitis (227.40±142.13 pg/mL). The difference was statistically significant (p-value <0.005) [Table/Fig-1].

The comparison of haematological parameters and coagulation profile (PT) of cases and controls are given in the [Table/Fig-2]. There was no statistically significant difference in RBC and WBC counts between two groups. But patients with alcoholic hepatitis had significantly low platelet count compared to ALD patients

S. No.	Variables	ALD patients		p-value
		With hepatitis (n=26)	Without hepatitis (n=26)	
1	RBC count (million/cumm)	3.29 (2.87-3.66)	3.3 (2.72-3.99)	0.784
2	WBC count (×10³ µL)	8.5 (4.93-12.5)	7.84 (5.66-9.13)	0.611
3	Platelet Count (×10³ µL)	128.5 (98-157)	191.5 (99.5-254)	0.034*
4	Prothrombin Time (PT) (Sec)	20.4 (16.95-24.45)	16.55 (14.18-20.78)	0.019*

[Table/Fig-2]: Comparison of haematological parameters between Alcoholic Liver Disease (ALD) patients with or without hepatitis.
Data are expressed as median (Inter quartile range); Mann-Whitney U test (non parametric) was used to compare; *p-value <0.05 considered as statistically significant; ALD: Alcoholic liver disease; RBC: Red blood cell; WBC: White blood cell

without hepatitis (p-value=0.034). ALD patients with hepatitis also had statistically significant high PT (p-value=0.019) compared to ALD patients without hepatitis. The low platelet count and elevated PT in patients with alcoholic hepatitis infer the disrupted coagulation profile in Alcoholic hepatitis patients.

Child-Pugh (CP) score: [Table/Fig-3] shows the categorisation of entire study population based on CP score. In moderately impaired group, subjects were more in without hepatitis group. A 61% of ALD patients with hepatitis had advanced hepatic dysfunction.

CP score	Class A (n=9)		Class B (n=22)		Class C (n=21)	
	With hepatitis	Without hepatitis	With hepatitis	Without hepatitis	With hepatitis	Without hepatitis
Mean±SD	6	5.38±0.48	7.89±0.99	8±0.88	11.44±1.00	10.8±0.75
No. of study participants	1 (4%)	8 (31%)	9 (35%)	13 (50%)	16 (61%)	5 (19%)

[Table/Fig-3]: Distribution of participants based on Child-Pugh (CP) score categories.
CP score is expressed in Mean±SD; Categorical data is expressed as frequency and percentage, n (%)

Correlation analysis of liver parameters with TNF-α: Spearman's correlation analysis was carried out to find out the association between TNF-α and other liver parameters, such as bilirubin, AST, ALT, and ALP, among cases and controls separately. There was no statistically significant correlation between liver parameters and TNF-α, although RBC count showed a positively correlated with TNF-α. The results are given in [Table/Fig-4].

S. No.	Parameters	TNF-α			
		With hepatitis		Without hepatitis	
		r-value	p-value	r-value	p-value
1	AST	0.185	0.364	-0.057	0.783
2	ALT	0.147	0.473	-0.262	0.196
3	ALP	-0.071	0.731	0.009	0.966
4	Bilirubin	0.076	0.711	0.130	0.527
5	T. Protein	0.133	0.516	0.285	0.158
6	Albumin	0.154	0.452	-0.118	0.566
7	RBC count	0.395	0.046*	-0.068	0.741
8	WBC count	0.099	0.627	-0.122	0.551
9	Platelets	-0.038	0.858	-0.034	0.870
10	Prothrombin Time (PT)	-0.170	0.406	0.115	0.577

[Table/Fig-4]: Correlation analysis of TNF-α with liver and haematological parameters among cases and controls.
Spearman's correlation (r) was used to find out the relation between TNF-α and liver biomarkers; RBC: Red blood cell; WBC: White blood cell

Correlation analysis was carried out to find out the association between CP score with other liver parameters and TNF-α. The total bilirubin levels have positive correlation with CP score in both cases and controls. The association was statistically significant as total bilirubin is one of the components to calculate CP score (p-value <0.05). CP score had significant positive correlation with PT in both ALD patients, with and without hepatitis group. There was significant negative correlation with CP score with RBC count in ALD patients without hepatitis group. The levels of TNF-α and other liver parameters, such as AST, ALT, and ALP, did not have any correlation with CP score. The results are shown in [Table/Fig-5].

S. No.	Parameters	Child-Pugh (CP) score			
		With hepatitis		Without hepatitis	
		r-value	p-value	r-value	p-value
1	AST	0.375	0.059	0.338	0.091
2	ALT	0.334	0.096	0.250	0.218
3	ALP	0.296	0.143	-0.149	0.469
4	Bilirubin	0.759	0.000*	0.698	<0.001*
5	TNF-α	-0.049	0.812	0.196	0.337
6	Total protein	0.140	0.494	-0.085	0.680
7	Albumin	-0.145	0.480	-0.676	<0.001*
8	RBC count	0.183	0.371	-0.497	0.010*
9	WBC count	0.274	0.175	0.034	0.868
10	Platelets	-0.118	0.574	-0.388	0.050
11	Prothrombin Time (PT)	0.710	<0.001*	0.566	0.003*

[Table/Fig-5]: Correlation analysis of Child-Pugh (CP) score with liver and haematological parameters, and TNF-α among cases and controls.
Spearman's correlation (r) was used to find out the relation between Child-Pugh (CP) score with liver, haematological parameters and TNF-α; *p-value <0.05 considered statistically significant; RBC: Red blood cell; WBC: White blood cell

DISCUSSION

This study was carried to estimate the level of TNF-α in ALD with or without hepatitis and to correlate it with other liver function parameters. In current study, significant changes were observed in platelet count and PT. Patients with alcoholic hepatitis showed low platelet count and high PT when compared to patients with ALD without hepatitis. This result showed abnormality of synthetic function of liver with respect to clotting factor.

This study results showed significant elevation of AST than ALT in alcoholic hepatitis. The alcohol induced mitochondrial injury is the reason for elevation of AST more than ALT. Significant low level of albumin (2.5 gm/dL) in our study also proves the compromised liver function in ALD with hepatitis. Protein-energy malnutrition in alcoholic hepatitis patients is one of the factors in predicting severity and mortality [19]. ALP in patients with alcoholic hepatitis was higher than the patients without hepatitis, which could be due to intrahepatic cholestasis as a result of inflammation of liver tissue.

The CP score of alcoholic hepatitis patients were significantly higher than the patients without hepatitis. Around 76% of alcoholic hepatis patients had advanced liver dysfunction, as they have high CP score. The importance of TNF-α in disease progression was emphasised in a study by Kawaratani H et al., in an animal study [11]. The results of their study demonstrated that giving TNF-α-knockout mice too much alcohol did not harm their livers in either Non Alcoholic Steatohepatitis (NASH) or ALD. The study results confirm the role of TNF-α in causing liver cell injury. TNF-α acts as pro-inflammatory cytokine, which have gross effect on vascular system by increasing vascular permeability and vasodilation [20]. Animal studies using TNF-α inhibitor or TNF Receptor 1-deficient mice showed protection against alcohol-induced liver injury [21,22].

Even though animal studies showed promising evidence for the role of TNF-α-induced liver cell injury, the use of TNF-α fusion protein, Etarnacept as inhibitor of TNF-α failed to produce protective effect against liver cell injury in multicentric clinical trial done by Boetticher NC et al., [23]. Instead, TNF-α inhibitor increased susceptibility to infection and caused significantly higher mortality rate after six months of treatment. The possible reason could be that TNF-α is involved in the processes that result in liver injury and hepatocyte death, as well as in the processes that lead to liver regeneration and innate immunity. This raised concern in blocking the action of this cytokine and showed the importance of standardisation in the treatment of cytokine inhibitors. The possible reason for mortality in long-term treatment with TNF-α inhibitor of TNF-α believed to be renal failure as a complication of infections, because TNF-α contributes to immune cell functions in combating infections against mycobacteria and atypical organisms [23].

This current study results showed that patients with alcoholic hepatitis have high level of TNF-α compared to patients without hepatitis. This is in consistent with the results of study Sowa JP et al., which showed that elevated TNF-α levels in ALD patients with cirrhosis compared to ALD patients without cirrhosis. The same study also showed that the patients with ALD had higher levels of TNF-α than those with NAFLD [12].

A recent study by Aishwarya AA et al., also showed that there is evidence of increased levels of pro-inflammatory cytokine TNF-α in ALD patients compared to controls, and also TNF-α increased with age in patients with ALD [13]. The probable reason for elevation of inflammatory cytokine is the immune response against alcohol-induced liver cell injury. and the inflammation because of which kupffer cells, which are specialised immune cells produced in the liver are activated and initiate series of events which end up in damaging the liver cells. This cascade of events also stimulates the production of hepatic stellate cells, which are responsible for fibrosis of liver by synthesising extra cellular matrix proteins [24].

The effect of TNF-α on liver cells depends on its concentration. This was recently shown by the animal study done by Zhao S et al., [25]. Their study results showed that low concentration of TNF-α did not show any effect on liver enzymes and instead it promoted the proliferation of animal However liver cells. But, high concentration of TNF-α increased the level of liver enzymes and increased the level of Annexin V and Propidium Iodide-positive cells, which clearly showed liver cell death [25].

The metabolites of alcohol itself can cause direct liver cell injury. Furthermore, it is thought that intestinal microbial dysbiosis, bacterial translocation, and elevated lipopolysaccharide concentrations in the portal blood cause disruption of the gut's epithelial barrier, which in turn triggers the liver's innate immune response and increases the production of pro-inflammatory cytokines like TNF-α [26].

Limitation(s)

As the current study was a cross-sectional study, it could determine only associations between TNF-α levels and ALD with hepatitis versus non hepatitis, but it cannot establish a direct causal relationship. A relatively small sample size of 26 in each group may limit the generalisability of the findings. Additionally, TNF-α levels were assessed only at a single point in time, which was unable to identify the dynamic fluctuations influenced by disease progression or treatment interventions.

CONCLUSION(S)

From the results of current study, it can be concluded that the patients of ALD have elevation of TNF-α levels, when they progress to or have episodes of active hepatitis. TNF-α is responsible for immune system-mediated inflammation and liver cell injury. Therefore, estimation of TNF-α in ALD with hepatitis would help in providing knowledge about severity The above observation warrants further investigation of the potential of TNF alpha inhibitors in the treatment of alcoholic hepatitis.

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