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Analysis of certain clinical characteristics of hepatitis delta virus (HDV) patients

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Abstract

Acute hepatitis D (delta) is a chronic liver disease caused with co-infection of hepatitis D virus (HDV) and hepatitis B virus (HBV). In this study, blood serum samples are screened for HBV and HDV infections. The 4 confirmed out of 14 patients with real-time PCR for HDV infected were subjected for further comparative clinical analysis. Among the patients, the hemoglobin (17.18±0.149g/dL) and HCT (50.90±0.235%) levels increased significantly than standard reference values. Similarly, proline and flavonoids also observed higher, while *alanine aminotransferase* (ALT) and antioxidants decreased ($p \leq 0.05$) in the patients than normal reference values for the healthy person. These facts could be developed due to HDV stress on the body. The HDV patients showed inversely proportional relations in the levels of hemoglobin and platelets. These differential parameters could be improved with the good nutrients or supplements, which may be helpful for the survival of these patients. This present study could be helpful proper maintenance of HDV patients under HDV treatment.

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Introduction

The hepatitis D virus (HDV) is being a chronic human pathogen. Its genome is comprised on single negative-stranded RNA with 1.7kb size (Hughes, Wedemeyer, and Harrison, 2011; Pascarella and Negro, 2011). It is considered as being a defective virus unable to complete its life cycle without hepatitis B virus (HBV). The genome multiplication of HDV depends on an association with HBV (Huang and Lo, 2014; Wen and Wang, 2009). Acute chronic form of HBV arises among the HB patients with the existence of HDV (Oo and Mutimer, 2015). The mutual viral super-infection has a scarcity of global data burden for HDV infection (Negro, 2014; Rizzetto, 2015). According to WHO (WHO, 2017), globally in an approximate 257 million of chronic HBV patients are co-infected with HDV, which has been resulting in 15-20 million HBV patients infected with HDV.

The transmission routes of HDV are same as for HBV like as percutaneous or sexually, while vertical transmission is rare (Seo *et al.*, 2015). The HBV-vaccination prevents HDV coinfection, but after development HDV the HBV accelerates its progression to 70-90% faster in cirrhosis and fibrosis than HBV alone in all aged patients (Spearman *et al.*, 2017). In spite of that both HBV and HDV are genetically highly diverse viroid (Le Gal *et al.*, 2017; Spearman *et al.*, 2017) like as the HBV is proposed into nine genotypes (Kramvis, 2004), while HDV strains with eight distinct clades (Le Gal *et al.*, 2006; Radjef *et al.*, 2004).

The HBV is highly prevalent, while in various areas of the world it has been considered most importantly that HBV is transmitted at the early childhood stage because of cultural affiliations and ways to handle the nourishing children. It is the major rout of infectious transmission of HBV. With the passage of time children grow into adults, which are the carriers of high proportions of chronic HBV. Its conversion and or into HDV super infections considerably involved to add-up higher chronic liver disease burden (Negro 2014; Yeung and Roberts 2001). In these areas, around 70% HBV B carriers are HDV infected (Han *et al.*, 2011).

This concurrent infection of HBV with HDV complicates the viral treatment as the applied regimens works against HBV infection while HDV replication retained as normal. Even HDV suppresses the replication rate of HBV up to undetectable levels (Lin and Kao, 2011; Livingston *et al.,* 2007; Nguyen *et al.,* 2009; Yang *et al.,* 2008). This complicate viral infection stage decreases the diagnosis as well as their co-genotyping (Malik *et al.,* 2012; Yu *et al.,* 2005). For the detection and analysis of such complex viral disease patients is not an easy task. It depends on the availability of skilled person for their selection and the modern tools for proper analysis. In Pakistan, there are very few research stations with proper skilled hands for the study of HDV super-infected samples. Accurate diagnose gives the better response for the managed treatment of these patients.

In this study, samples of HBV-infected patients are collected from the village and analyzed with RT-PCR for the presence of both HBV and HDV. The sera of HDV super-infected patients are subjected for certain clinical analysis like as complete blood analysis. The estimation of these serum based parameters might be helpful in the management as well as the conversion of HDV super-infection from adults to children. Even fluctuations in these characters of serum are based on mode of their nutrition which may cause the imbalanced hormonal biosynthesis. By keeping these views under considerations, the serum is subjected to evaluate the intensive hematological parameters of HDV-superinfected patients against normal reference values.

Materials and methods

Collection of specimens

The fresh blood serum samples of selected HBV patients were collected in standard vials [anti-coagulated with EDTA-K_2 (ethylene di-amine tetra-acetic acid-dipotassium] from the apparently selected healthy individuals who have visited the routinely blood diagnostic laboratory. From this above collection, four patient's samples were selected, which are infected with chronic HDV. The blood samples were centrifuged and plasma/serum stored -20°C until its next use for the HDV diagnosis.

Diagnostics of HDV patients

The samples of HDV patients were confirmed via Cobas TaqMan-PCR (TaqMan 48; Roche Diagnostics, Germany) by following procedure reported (Mederacke *et al.,* 2010). The nucleic acids extracted with automated Cobas AmpliPrep Instrument (Roche Diagnostics, Germany) by using the nucleic acid isolation kit (TINAI). The HDV RNA was quantified with primers specific for the hepatitis delta antigen locus with final product size of 71-bp (Le Gal *et al.,* 2005).

Hematological analysis of HDV patients

Various hematological parameters were analyzed in the serum blood of HDV confirmed patients in comparison to normal healthy persons. The parameters including CBC (complete blood count), hematocrit (HCT), Hb level, mean cell Hb (MCH), MCH concentration (MCHC), mean cell volume (MCV) and red blood cell distribution width were determined with Hematology Analyzer (Model-Advia 2120, Bayer Diagnostics, USA) and the liver marker i.e. *alanine aminotransferase* (ALT) determined with Beckman Coulter Automatic Biochemical Analyzer by following their procedure given in the manuals (Haq *et al.,* 2018; Harthoorn-Lasthuizen *et al.,* 1999; Mosca *et al.,* 2009; Wang *et al.,* 2017).

Estimation of hemoglobin

The hemoglobin was estimated by following the procedure as already reported (LEWIS *et al.,* 1991; Shah *et al.,* 2011). Shortly, exact 20µl blood mixed in Drabkin's reagent with 1:200. The mixture was mixed thoroughly at room temperature for 10 min. Its absorbance was taken at 530 against blank Drabkin's reagent. It is also used in hemochromogens (12g/dL) standard solution preparation.

Measurements of antioxidant activity

The total antioxidant activity (TAA) was measured in a reaction mixture, which was prepared as by mixing the 2mL Tween-80, 0.2mL ascorbic acid (10 mM ascorbic acid) and 0.2mL ferrous sulfate solution (1 mM $Fe_2(SO_4)_3$ with 0.1mL hemolysate (or 0.2mL plasma). The mixture was mixed thoroughly and incubated for 48 hours at 40°C. Exactly its 2mL mixed with 1mL TCA (20% trichloroacetic acid) than 1mL of its supernatant was poured with 2mL 0.8% TBA (thiobarbituric acid). Mixture was boiled for 15 min than after cooling to room temperature OD532 of upper aqueous phase was taken (Evenson and Carmack, 1979; Korotkova *et al.,* 2013; Sun *et al.,* 1988).

Determination of glycinebetaine

For the determination of glycinebetaine, sample was diluted with dH_2O in (0.5:1) in a test tube. Exact 1mL pottassium iodide (1M) mixed with sample and incubated for 15 min at 65°C. The OD365 of reaction mixture was read against blank (Valadez-Bustos *et al.,* 2016).

Quantification of flavonoids

The 1ml sample was taken in a test tube than following reageants were added in this order one by one as 60µl sodium nitrite (3%), 60µl aluminium chloride (5%) and 0.88mL NaOH (1 M). The reaction mixture was mixed at room temperature properly. The OD540 was taken against blank (dH_2O) with all other reagents (Stefova *et al.,* 2003).

Statistical Analysis

The experiment was comprised on 04 replicates per sample. The collected data of this study was computed with computer based software CoStat (version 3.03) CoHort software, Berkeley, USA. The significant mean values normal to HDV patients were subjected for Duncan Multiple Range (DMR) test at 5% (Behrens, 1997; Henley, 1983; Quinn and Keough, 2002).

Results and discussion

The hepatitis delta virus (HDV) infection relies on fore-infection of hepatitis B virus (HBV) for its progressive pathogenesis. The con-infection of these both viruses causes an aggressive virulent hepatitis. The detection of hepatitis D virus above to hepatitis B virus had remained a forgotten virus due to lack of public awareness as well as lesser medical interest and financial research supports (Rizzetto, 1983; Rizzetto *et al.,* 1980). Recently, various diagnostic tools have increased the interest in hepatitis D for its detection and to improve its therapy (Ahn and Gish, 2014; Rizzetto, 2015). As the HDV infection occurs in a simultaneous and systematic co-infection with HBV or this super-infection occurs in the high HBsAg

carriers (Polish *et al.,* 1993). The primary HDV serological diagnostic tests for HDV-RNA reported in liver tissue and HDAg with RIA and EIA kits (Hackman *et al.,* 1996; Rizzetto *et al.,* 1980).

In this study, a number of HBV patients subjected for screening while 4 confirmed patients with HDV (HBsAg positive). These patients selected and subjected for the hematological studies. The serum of each patient had taken 4 times (by skipping one day) in 8 days. Each of these sample per day considered as a replicate and in this way, total 4-replicates per patient were arranged. These samples of the retrospective study subjected to measure the comparative hematological parameters. In addition, it confirms the stringency of HDV seroprevalence among the chronic active HBV patients. Even acute HDV in HBsAg positive and high ratio of HBsAg positive had considered in chronic HDV-Super infection. The Hematocrit parameters shows blood disorders due its abnormal levels (Table 1) among the HDV patients than normal.

Table 1. Comparative analysis of hematological (complete blood count) parameters of the confirmed hepatitis D virus patients in comparison to standard normal reference values for healthy persons.

SN	Parameters	Patient - I	Patient - II	Patient - III	Patient - IV	Significances
01.	RBCs (10^{12}/L) *(3.46-5.07)*	^{a}6.053±0.064	^{b}4.953±0.076	^{c}5.090±0.056	^{b}5.478±0.064	25.57***
02.	HB (g/dL) *(13.20-16.3)*	^{a}17.18±0.149	^{c}15.33±0.397	^{b}12.73±0.531	^{c}15.95±0.299	56.48***
03.	MCV (fl) *(66.06-95.60)*	^{b}83.95±0.659	^{a}87.65±0.494	^{c}78.80±0.612	^{b}84.23±0.293	46.76***
04.	HCT (%) *(41.9-48.7)*	^{a}50.90±0.235	^{c}44.48±0.613	^{d}41.15±0.210	^{b}45.73±0.243	122.8***
05.	MCH (pg) *(21.10-31.23)*	^{a}28.95±0.222	^{b}27.00±0.365	^{c}25.38±0.214	^{a}28.35±0.233	36.73***
06.	MCHC (g/dl) *(28.70-34-60)*	^{a}34.15±0.290	^{a}34.15±0.350	^{b}31.73±0.131	^{a}34.35±0.210	23.29***
07.	NEU (%) *(4.0-10.0)*	^{b}47.23±0.364	^{c}46.25±0.194	^{d}43.38±0.229	^{a}51.40±0.227	161.2***
08.	MO (%) *(4.40-12.13)*	^{b}9.575±0.165	^{c}07.35±0.104	^{a}13.13±0.165	^{d}5.575±0.111	548.0***
09.	EOS (%) *(1-6)*	^{b}02.55±0.065	^{a}04.40±0.220	^{c}01.40±0.183	^{b}02.55±0.144	57.78***
10.	LY (%) *(20.27-55.48)*	^{b}39.70±0.402	^{a}41.60±0.208	^{a}42.33±0.202	^{b}39.38±0.272	25.59***
11.	BASO (%) *(0-1)*	^{a}0.600±0.041	^{bc}0.275±0.048	^{c}0.175±0.048	^{b}0.400±0.041	16.95***
12.	WBCs (10^{9}/L) *(3.80-11.20)*	^{b}7.150±0.133	^{c}5.025±0.165	^{d}4.150±0.144	^{a}8.175±0.175	142.3***
13.	PLT (10^{8}/µL) *(150-400)*	^{b}152.0±2.646	^{d}88.00±2.646	^{c}129.0±2.160	^{a}179.5±2.784	226.9***
14.	ALT (U L^{-1}) *(35 IUmL^{-1} - ♂)*	^{a}24.5±8.312	^{ab}18.75±6.447	^{b}18.0±1.581	^{ab}20.0±5.831	2.926^{ns}

CBC: Complete blood count, RBCs: Red blood cells, HB: Hemoglobin, MCV: Mean corpuscular volume, HCT: Hematocrit, MCH: Mean carpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, NEU: Neutrophillus, MO: Monocytes, EOS: Eosinophillus, LY: Lymphocytes, BASO: Basophillus, WBCs: White blood cells, PLT: Platelets, ALT: Alanine aminotransferase, ♂: Male symbol, Ref: Reference.

Data is collected in four replicates of the same patient and expressed as mean ± SE (n = 4). The Duncan's Multiple Range test of significant parameters also calculated ($p \leq 0.05$).

The CBC of the selected confirmed patients (all were male) has shown significant variation for HB, HCT, RBC count, PLT count and absolute monocyte count (Table 1). The control reference values (RVs) of the respected parameter like as HB, HCT and RBC were lower while higher for the PLT counts. The non-CBC parameters including serum iron and ferritin observed lower but transferrin remained higher (Fig 1). In CBC, a negative correlation has observed between the HB levels and platelet count.

The CBC parameters are indicating that patient-I have higher HB (17.18±0.149), including other patient-II and patient-IV (Table 1). It means that HDV patients are anemic apparently as the Hb concentrations are observed significantly different from normal ones to patients (Vos *et al.,* 2011).

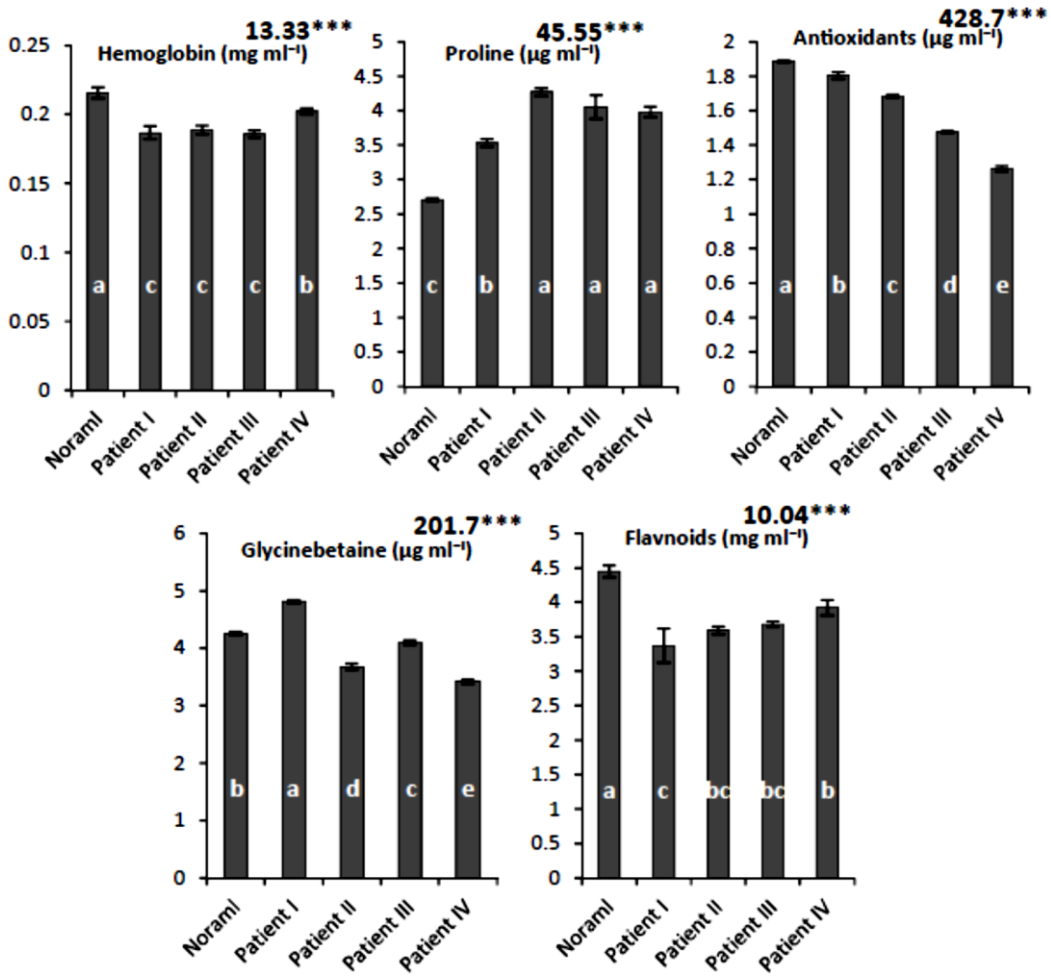

Fig. 1. Analysis of certain biochemical parameters existed among the HDV patients in comparison to standard normal reference values for healthy persons.

The HDB patients shows abnormal values of all hematological traits which are observed in their CBC. Abnormal values of HB, HCT, MCV and MO in patients showed acute liver failure including various renal complications well (Chakravarti *et al.* 2017). These fluctuations among the serological parameters may be associated with the other cell fibrosis among the body organs including liver, kidney etc. The low to higher ALT values of the HDV patients have strong association for the proper detection of stage of liver fibrosis and rate of inflammation (Table 1). The patients affected with HDV showed decreased ALT activities than the fibrosis normal or reference (35 $IUmL^{-1}$ for male) to abnormal mild-moderate (below than 35 $IUmL^{-1}$) and abnormal severe ranges observed in patient I and IV as shown in table 1 (Ho *et al.,* 2013). In critical with the support of the results from CBC to serological findings have shown that both HBV and HDV coinfection increasingly causes liver disease (Yurdaydın *et al.,* 2010).

The delay in the diagnosis as well as treatment of HDV patients increases the level of cell fibrosis in liver including heart also. It is the very difficult increasing stage of the HDV patients with undesirable potential effects of HDV over HBV management for the purpose to save the life of such patients. This untolerateable and unmanageable relationship among the HDV and HBV has also been indicated through mathematical model (Xiridou *et al.,* 2009).

The cure of HBV becomes much difficult with induction of HDV. The abnormal level of liver cell fibrosis in HDV is potential life threat for the patient.

Conclusions

The purpose of present study locates the high prevalent area of the country and also to bring the attention of the researchers and health associated agencies on the burning issue. The hepatitis B and D viral coinfection increase the cell fibrosis severity in liver. The HDV infection moderately alters the levels of RBC, HCT, HB and lymphocytes among the respondents significantly. The HDV patients are surviving under anemia, bacteremia and other inflammatory etc risks.

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