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Investigation of Selective Biochemical Markers from Chronic Hepatitis C Patients in Relation to Environmental Pollutants

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ABSTRCT: Hepatitis C virus (HCV) causes acute and chronic hepatitis which can eventually lead to permanent liver damage, hepatocellular carcinoma and death. The interactions among chronic liver disease and essential micronutrients in blood are arguable and have not been well understood. Therefore, HCV was analyzed in the serum from 35 previously untreated patients with chronic hepatitis C. In serum of all subjects different biochemical parameters were analyzed (Sodium, Potassium, Iron, Zinc, Phosphorus, Calcium, CRP, ALT, AST, Amylase, GGT, Cholesterol, TG, HDL, LDL and Serum Protein). The control group contained 15 healthy volunteers. Industrial localities and other sources that emanate various metals to the environment are known to induce metal toxicity to the population through a number of routes. All patients and healthy individuals investigated by using recommended procedures. The contents of zinc, calcium, cholesterol, triglyceride and total protein were significantly lower in hepatitis C patients than in the controls. On the contrary potassium, phosphorus, C-reactive protein, alaninie aminotransferase, aspartate amino transferase, amylase and g-glutamyl transferase was significantly higher than normal controls. Furthermore, serum sodium, iron, high density lipoproteins and low density lipoproteins remained non-significant.

Key words: Hepatitis C virus · Trace elements · Liver enzymes · Lipid profile

INTRODUCTION

The essential metals at trace levels play vital role when present in human body and may cause several disorders when present beyond specific concentrations [1]. These elements have certain limits for normal functions of a body and balance of these constituents may be affected due to various types of pollutants. Lower or higher concentration of these elements in body adversely affects the normal biological functions. Metals such as Co, Cu, Fe, Mn, Ni and Zn are essential for biological system but chronic metabolic disturbances may occur due to deficiency or excess of these metals [2]. Metals and heavy metals may be perilous to human health for numerous reasons. They increase the formation of extremely reactive particles known as free radicals, which damage cell membranes, interfere with standard cellular functions, cause disease and aging. Iron, lead, mercury, cadmium and arsenic are of the greatest concern because they interfere with the electron transfer process, thereby causing free radical oxidation and damage. Metals also cause damage by forming molecular bonds with enzymes and antioxidants such as glutathione, thereby preventing normal enzyme and antioxidant reactions. Oxidative damage to liver cells is known to be promoted in the presence of metals in varying physiologic concentrations. Serum trace elements levels and their ratios are frequently reported to be a good marker for diagnosing various diseases. The relationship between chronic liver disease and trace elements has not been clearly understood no defined data are available about the trace heavy metals, especially Co and Ni levels in viral liver disease patients. HCV is one of several viruses that can cause hepatitis [3]. Among body parts, blood is measured as the most dependable restraint for the estimation of exposure of metal pollutant in wide-ranging population, because these metals reach different parts of body through blood circulation and cause serious damages [4].

In hepatitis C patients, serum zinc levels and the zinc/copper ratios were considerably higher in complete responders than in non-responders to IFN therapy. Zinc is a constituent of a number of enzymes and as such is involved in a large number of metabolic processes. Zinc exerts a protective action on liver cell activity by

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possibly preventing cellular damage caused by oxidative stress in chronic hepatitis or liver cirrhosis [5]. According to Nielsen, non-essential elements such as Pb and Cd are considered toxic and their presence in the body can cause profound biochemical and neurological changes in the body even at ultra-trace level [6]. In addition to these constituents, human body contains several other elements and complex compounds, which influence many normal body functions. These elements are assimilated through food, water and the environment [7]. Several findings have been reported for the level of Fe, Cu, Zn and Pb in blood serum of patients with hepatitis C virus, but very few for Co and Ni. Serum iron status of patients with various forms of hepatitis and cirrhosis of the liver was studied, as well as the correlation between the degree of hepatocyte damage and the status of serum iron parameters [8].

The low serum zinc level is common in patient with liver cirrhosis due to decreased intake, decrease absorption, decreased bioavailability and increased losses (because of malabsorption). There is also reduced liver protein synthesis in patients with liver cirrhosis, the metallothionein [MT] is an important zinc-binding protein (formed by liver) and is involved in zinc metabolism, homeostasis and its release in number of oxidants, the released zinc will inhibit the activity of the enzymes involved in fibrogenesis in the liver, all these are yet known pathophysiological mechanisms [9]. Clinical studies reported that HCV related chronic liver disease patients at different stages of liver damage have impaired metabolism of trace elements. Of those trace elements, serum copper and zinc levels have been reported to be highly sensitive in the diagnosis of some cancers and hepatic disorders [10].

MATERIALS AND METHODS

Experimental Design: A total number of 50 individuals; (15 healthy and 35 hepatitis C virus) including in this research project have been selected; we have selected the chronic Hepatitis C and healthy subjects having a background of industrial employment. Selected individuals were divided into two groups i.e. Group I: Control: Healthy Persons and Group II: Test: Hep C Patients.

Locations: Industrial workers have been selected from various localities of Faisalabad dominantly working in industries and living in their vicinity.

Patients and Healthy Donors: All patients and healthy individuals have been selected. Diagnosis of hepatitis based on symptoms as well as blood tests; to assess the scope of selective vital biochemical parameters and micronutrients in serum of patients with chronic hepatitis C. The healthy individuals were without any sign and symptoms of hepatitis, while their blood tests were too negative for HCV.

Blood Sample Collection and Preparation: Blood samples (5 mL each) of patients have been collected by venous puncture from hospital; in Aziz Fatimah Hospital and Liver Centre of Civil Hospital Faisalabad. Blood samples of healthy donors have been collected from the same periphery like blood donors. Disposable syringes have been used for blood sampling. The blood sample left standing for 1 hour to coagulate; serum separated at 45000 rpm centrifugation for 10 min, transferred to 5 ml polystyrene tube and stored at -39°C till further analysis.

Analysis: The present study instigated to evaluate the Sodium, Potassium, Iron, Zinc, Phosphorus, Calcium, CRP, ALT, AST, Amylase, GGT, Lipid profile (Cholesterol, Triglycerides, HDL, LDL) and serum protein concentrations in sera from viral hepatitis patients.

HCV: HCV determined by state of the art special chemistry, immunology and hormone analyzer; AxSYM system by Abbott Diagnostics, USA. The system detects HCV antibodies qualitatively by HCV version 3.0 microparticle enzyme immunoassay technology. The biological principle of the procedure; AxSYM HCV version 3.0 is based on the microparticle enzyme immunoassay technology [11].

Sodium and Potassium: Sodium and potassium analysis done on 9180 fully automated electrolyte analyzer (microprocessor-based technology) by Roche Diagnostics, working on the principle of ion selective electrodes [12].

Iron: Iron analysis done on Hitachi-902 fully automated chemistry analyzer by Roche diagnostics. Under acidic conditions, iron is liberated from transferrin. Lipemic samples are clarified by the detergent. Ascorbate reduces the released Fe^{3+} ions to Fe^{2+} ions which then react with Ferrozine to form a colored complex. The color intensity is directly proportional to the iron concentration and can be measured photometrically [13].

Zinc: Zinc analysis done on Microlab-300 semi-automated chemistry analyzer by Merck diagnostics. Test principle: Zinc in a pH 8.60 buffer system, forms with specific complexant 5-Br-PAPS a stable coloures complex. The interferences, due to oligoelements present in the sample, are eliminated using particular reaction condition and specific masking agents [14].

Phosphorus: Phosphorus analysis done on Hitachi-902 fully automated chemistry analyzer by Roche diagnostics. Test principle; assay for the quantitative in vitro determination of phosphorus in human serum and plasma with Roche automated clinical chemistry analyzer. Inorganic phosphate forms an ammonium phosphomolybdate complex having the formula $[NH_4]_3\{PO_4[MoO_3]_{12}\}$ with ammonium molybdate in the presence of sulfuric acid. The complex is determined photometrically in the ultraviolet region 340 nm [15].

Calcium: Calcium analysis done on Hitachi-902 fully automated chemistry analyzer by Roche diagnostics. Test principle; in vitro test for the quantitative determination of calcium in human serum, plasma and urine on Roche automated clinical chemistry analyzer. The color intensity of the purple complex formed is directly proportional to the calcium concentration and is measured photometrically [16]

C-Reactive Protein (CRP): CRP performed by immunoturbidimetric assay on Hitachi-902 fully automated Chemistry analyzer by Roche diagnostics. Test principle; Immunoturbidimetric assay for in vitro quantitative determination of CRP in human serum and plasma on Roche automated clinical chemistry analyzers. C-reactive protein is the classical acute phase protein to inflammatory reactions. The Roche CRP assay is based on the principle of particle-enhanced immunoturbidimetric agglutination assay. Anti-CRP antibodies coupled to latex microparticles react with antigen in the sample to form an antigen/antibody complex that is measured turbidimetrically [17].

ALT/SGPT: ALT/SGPT analyzed on Hitachi-902 fully automated Chemistry analyzer by Roche diagnostics. In vitro test for the quantitative determination of alanine amino-transferase in human serum and plasma on Roche automated clinical chemistry analyzer. ALT is the enzyme which catalyzes this equilibrium reaction. The pyruvate increase is measured in a subsequent indicator reaction which is catalyzed by lactate dehydrogenase. In the second reaction, NADH is oxidized to NAD. The rate of decrease in NADH is directly proportional to the rate of formation of pyruvate and thus ALT activity [18].

AST/SGOT: AST/SGOT analyzed on Hitachi-902 fully automated Chemistry analyzer by Roche diagnostics. In vitro test for the quantitative determination of aspartate aminotransferase in human serum and plasma on Roche automated clinical chemistry analyzer. The enzyme AST catalyzes this equilibrium reaction. The increase in oxaloacetate is determined in an indicator reaction catalyzed by malate dehydrogenase. NADH is oxidized to NAD^+. The rate of the photometrically determined NADH decreas is directly proportional to the rate of formation of oxaloacetate and thus AST activity [19].

Amylase: Amylase analyzed on Hitachi-902 fully automated Chemistry analyzer by Roche diagnostics. Enzymetic in vitro test for the quantitative determination of á-amylase in human serum, plasma and urine on Roche automated clinical chemistry analyzer. The color intensity of the p-nitrophenol formed is directly proportional to the á-amylase activity and is measured photometrically [20].

GGT (Gamma Glutamyl Transferase): GGT analyzed on Hitachi-902 fully automated Chemistry analyzer by Roche diagnostics. In vitro test for the quantitative determination of gamma-glutamyl transferase in human serum and plasma on Roche automated clinical chemistry analyzer. Gamma-glutamyltransferase transfers the ã-glutamyl group of L- ã-glutamyl-3-carboxy-4-nitroanilide to glycylglycine. The amount of 5-amino-2-nitrobenzoate liberated is proportional to the GGT activity and can be measured photometrically [21].

Cholesterol: Cholesterol analyzed on Hitachi-902 fully automated Chemistry analyzer by Roche diagnostics. Enzymatic in vitro test for the direct quantitative determination of cholesterol in human serum and plasma on Roche automated clinical chemistry analyzer. Cholesterol esters are cleared by the action of cholesterol esterase to yield free cholesterol esterase to yield free cholesterol and fatty acids. Cholesterol is converted by oxygen with the aid of cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide created forms a red dyestuff by reacting with 4-aminophenazone and phenol under the catalytic action of

peroxidase. The color intensity is directly proportional to the concentration of cholesterol and can be determined photometrically [22].

Triglycerides: Triglycerides analyzed on Hitachi-902 fully automated Chemistry analyzer by Roche diagnostics. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff. The color intensity is directly proportional to the concentration of cholesterol and can be determined photometrically [23].

LDL (Low Density Lipoproteins): LDL analyzed on Hitachi-902 fully automated Chemistry analyzer by Roche diagnostics. Homogenous enzymatic in vitro assay for the direct quantitative determination of LDL-cholesterol in human serum and plasma on Roche automated clinical chemistry analyzer. Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ^4 cholestenone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-aminoantipyrine and HSDA to form a purple-blue dye. The color intensity of this dye is directly proportional to the cholesterol concentration and is measured photometrically [24].

HDL (High Density Lipoprotein): HDL analyzed on Hitachi-902 fully automated Chemistry analyzer by Roche diagnostics. Enzymatic in vitro assay for the direct quantitative determination of HDL-cholesterol in human serum and plasma on Roche automated clinical chemistry analyzer. Test principle; Homogeneous enzymatic colorimetric test. In the presence of magnesium ions, dextran sulfate selectively forms water-soluble complexes with LDL, VLDL and chylomicrons which are resistant to PEG-modified enzymes. The cholesterol concentration of HDL-cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approx. 40 %). In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-aminoantpyrin and HSDA to form a purple-blue dye. The color intensity of this dye is directly proportional to the cholesterol concentration and is measured photometrically [25].

Total Protein: Total protein analyzed on Hitachi-902 fully automated Chemistry analyzer by Roche diagnostics. In vitro test for the quantitative determination of total protein in human serum and plasma on Roche automated clinical chemistry analyzer. Divalent copper reacts in alkaline solution with protein peptide bonds to form the characteristic purple-colored biuret complex. Sodium potassium tartrate prevents the precipitation of copper hydroxide and potassium iodide prevents autoreduction of copper. The color intensity is directly proportional to the protein concentration which can be determined photometrically [26].

Statistical Analysis: Data has been expressed as mean ± SE. Significant differences in variables between two groups have been tested by t-test. A linear regression has been used to analyze the correlation among variables [27].

RESULTS AND DISCUSSION

A total number of 50 subjects; [fifteen healthy and thirty five hepatitis C virus] including in this research project have been selected; the selected subjects with chronic Hepatitis C having a background of industrial employment and healthy subjects from different sites. Selected persons have been divided into two groups.

- Group I: Control: Healthy Persons
- Group II: Test: Hep C Patients

HCV determined by state of the art special chemistry, immunology and hormone analyzer; AxSYM system by Abbott Diagnostics, USA. The system detects HCV antibodies qualitatively by HCV version 3.0 microparticle enzyme immunoassay technology [HCV on AxSYM by Abbott. kit brochure, 2008]. As we have selected 35 those subjects which were HCV positive and are without interferon treatment. Then these samples were analyzed for biochemical parameters, the values of specific micronutrients in serum of hepatitis C patients and healthy subjects were estimated.

In the present investigation, the contents of zinc, calcium, cholesterol, triglyceride and total protein were significantly lower in hepatitis C patients compared to healthy subjects. On the contrary potassium, phosphorus, C-reactive protein, alaninie aminotransferase, aspartate amino transferase, amylase and g-glutamyl transferase was significantly higher than normal controls.

Furthermore, serum sodium, iron, high density lipoproteins and low density lipoproteins differed not much and remained non-signficant.

In serum of all subjects different biochemical parameters were analyzed (Sodium, Potassium, Iron, Zinc, Phosphorus, Calcium, CRP, ALT, AST, Amylase, CRP, GGT, Cholesterol, TG, HDL, LDL and serum protein) were determined as biochemical markers. The control group contained 15 healthy volunteers. Industrial localities and other sources that emanate various metals to the environment are known to induce metal toxicity to the population through a number of routes. All patients and healthy individuals investigated by using recommended procedures.

There was no significant change in sodium as the values in HCV patients and healthy subjects are mean±SE [group1] 140.69±0.377, mean±SE [group2] 139.93±0.589; Potassium [K] in the patients was increased significantly than that in the healthy controls, values are mean±SE [group1] 4.30±0.058, mean±SE [group2] 3.95±0.057; There was no significant change in iron as the values in HCV patients and healthy subjects are mean±SE [group1] 81.31±7.238, mean±SE [group2] 78.93 ±3.979; Zinc [Zn] concentration in the patients was decreased than that in the healthy controls values in HCV patients and healthy subjects are mean±SE [group1] 66.77±1.631, mean±SE [group2] 87.73±1.774; Phosphorus [P] in the patients was increased than that in the healthy controls values in HCV patients and healthy subjects are mean±SE [group1] 4.47±0.091, mean±SE [group2] 3.49±0.130; Calcium [Ca] in the patients was decreased than that in the healthy controls, values in HCV patients and healthy subjects are mean±SE [group1] 7.75±0.152, mean±SE [group2] 9.44±0.192; C-Reactive Protein [CRP] in the patients was significantly increased than that in the healthy controls, values in HCV patients and healthy subjects are mean±SE [group1] 35.30±0.152, mean±SE [group2] 2.01±0.308; Homocysteine [Hcy] in the patients was increased than that in the healthy controls, values in HCV patients and healthy subjects were mean±SE [group1] 15.45±0.434, mean±SE [group2] 9.36±0.385; Alanine amino transferase [ALT] in the patients were significantly increased than that in the healthy controls, values in HCV patients and healthy subjects are mean±SE [group1] 51.80±4.141, mean±SE [group2] 23.87±1.486; Aspartate amino transferase [AST] in the patients was significantly increased than that in the healthy controls, values in HCV patients and healthy subjects are mean±SE [group1] 75.69±7.288, mean±SE [group2] 25.73±1.132; Amylase [Amy] in the patients was increased than that in the healthy controls, values in HCV patients and healthy subjects are mean±SE [group1] 73.11±5.312, mean±SE [group2] 55.60±3.008; Gamma glutamyl transferase [GGT] in the patients was increased than that in the healthy controls, values in HCV patients and healthy subjects are mean±SE [group1] 56.69±4.066, mean±SE [group2] 20.13±1.540; There was significant decreased values of Cholesterol in HCV patients than healthy subjects, values mean±SE [group1] 156.13±8.605, mean±SE [group2] 164.97±7.267; Triglycerides in the patients was decreased than that in the healthy controls, values in HCV patients and healthy subjects are mean±SE [group1] 125.57±9.769, mean±SE [group2] 180.13±21.026; there was no significant change in High density lipoprotein as the values in HCV patients and healthy subjects are mean±SE [group1] 28.11±1.782, mean±SE [group2] 35.07±4.081; there was no significant change in Low density lipoprotein as the values in HCV patients and healthy subjects are mean±SE [group1] 84.29±5.279, mean±SE [group2] 81.40±6.416; Total protein values in the patients were decreased than that in the healthy controls, values in HCV patients and healthy subjects are mean±SE [group1] 6.01±0.113, mean±SE [group2] 8.37±0.184 respectively and statistical tool indicates decrease in Total protein concentration of the patients [P<0.05] than healthy subjects.

Plasma concentrations of Zn and Fe in patients with chronic hepatitis C and healthy controls [mean±SE]

	Groups	Plasma
	Zn [mg/L]	Fe [mg/L]
Patients	0.13±0.01a	0.56±0.04
Healthy controls	0.55±0.06	0.57±0.03

P < 0.05 vs healthy controls [28].

The patients with hepatocellular carcinoma, serum values of trace elements are alike to those values of liver cirrhosis. In the patients of acute hepatitis, serum zinc, calcium and magnesium concentrations decline, while iron, phosphorus and copper concentrations boost. Huang *et al*. 2010 stated that the mean hs-CRP value of chronic hepatitis C patients was considerably raised than healthy controls [0.97 ± 0.11 vs. 0.24 ± 0.07 mg/L, P < 0.001] [29]. Most patients with significant amounts of fibrosis and histological activity had ALT levels greater than 1.5 times the upper limit of normal [30]. Elevated GGT levels were observed in 96 patients [48%] with chronic HCV infection. In fact, different rates of occurrence, ranging from 38.4% to 70%, have been published [31]. A study based on hospital cohorts that demonstrated the association between lower serum cholesterol levels and

HCV infection in plasma and the association between HCV infection and hypobetalipoproteinemia [32]. The serum cholesterol and triglyceride levels in anti-HCV-positive subjects with HCV RNA were significantly lower than in those subjects who were also anti-HCV-positive [33].

Therefore, the present results suggest that changes of zinc, calcium, cholesterol, triglyceride and total protein levels in serum of patients with chronic hepatitis C are directly related to the pathology developed in the liver. The outcome of HCV infection is also thought to depend on the balance between the rate of viral replication, rapidity and specificity and the effectiveness of the host immune response. In addition, higher levels of blood potassium, phosphorus, C-reactive protein, alaninie aminotransferase, aspartate amino transferase, amylase and g-glutamyl transferase are directly correlated with higher HCV and liver cirrhosis. Little is known about the possible regulatory mechanism of micronutrients in the pathogenesis of HCV. However, these nutrients are known to assist in immune-mediated response and involve in the alteration of virus Genomes.

CONCLUSION

The level of zinc, calcium, cholesterol, triglyceride and total protein in serum have important impact on the viral replication in chronic hepatitis C. The distribution of these parameters might be significant biomarkers for HCV. While potassium, phosphorus, C-reactive protein, alanine aminotransferase, aspartate amino transferase, amylase and g-glutamyl transferase were considerably raised and important parameters to check the prognosis of HCV and liver cirrhosis.

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