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Evaluates the Efficiency of Interferon-Alpha Therapy for Different Time Intervals on the Levels of Iron in Serum Samples of Chronic Hepatitis C Patients

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Abstract

It was investigated that the Interferon-α (IFN-α) therapy to patients with hepatitis C are correlated with iron levels in serum. The aim and objective of this study was to test whether the iron levels in blood serum samples were decreased after IFN-α therapy for different time intervals.

In present follow-up study, iron levels in serum samples of chronic Hepatitis C (HCV) patients before and after IFN-α therapy at different time interval (24-48 weeks) were evaluated. 160 females HCV patients were selected for follow-up study. Blood samples were collected from patients before and after treatment of IFN-α therapy. For comparative purpose, blood samples from healthy female subjects of the same age group were also collected. The serum iron was determined by electro thermal atomic absorption spectrometry, after microwave assisted acid digestion method. After INF-α therapy, HCV RNA level was undetectable in 32% and 58% patients, for 24-48 weeks, respectively. The level of Fe in serum samples were significantly reduced after longer period of treatment with INF-α (48 weeks).

Keywords: Iron; Hepatitis C; Interferon therapy; Serum; Biochemical parameters

Introduction

Chronic hepatitis C may result in progressive hepatic injury and fibrosis, sometimes resulting in cirrhosis and liver failure. This disease is a leading cause of liver-related morbidity and mortality in all over the world especially in Asian countries [1]. Iron (Fe) is an essential element for all living organisms, require for a wide range of metabolic processes including DNA synthesis, oxygen transport, and energy production. Whereas excess Fe can be harmful to the organisms, in part through the generation of oxygen radicals, and is potentially lethal [2].

Chronic infection with HCV virus has sometimes been associated with excess Fe deposition in the liver [3]. The liver is the main iron storage organ and it plays a fundamental role in iron metabolism. The iron transport protein, transferrin, and the major iron storage protein, ferritin, are synthesized in the liver [4]. There is a key link between iron metabolism and pathophysiology of viral hepatitis [5]. Increase in iron stores (increase in serum ferritin and transferrin iron saturation) leads to increased response to HCV infection, and progression of chronic hepatitis C [6]. The role of iron has been pointed out as an important element in the natural history of HCV. In fact, serum Fe stores are frequently increased in chronic HCV infected, but little is known about the significance of these abnormalities [7,8]. In experimental settings, excess hepatic Fe deposition is known to be hepatotoxic and may exacerbate liver injury in several ways [7]. First, Fe, which is essential for the growth of all organisms, may facilitate HCV replication [8]. Second, Fe may worsen liver tissue injury by increasing the formation of highly toxic hydroxyl radicals by the Fenton reaction, leading to progressive liver fibrosis [9] and increased risk of developing liver carcinoma [10]. It was reported that the Fe deposition in the liver of chronic HCV patients may be due to release of from damaged hepatocytes [11-13]. The association between HCV infection and altered serum Fe profile or hepatic Fe deposits has been evaluated in several studies [14] but, the prevalence of hepatic Fe overload, its relationship with the severity of disease and influence on the response to antiviral treatment are still controversial [15,16]. Metal levels in blood serum have been reported to be highly sensitive in the diagnosis of liver diseases [17]. Atomic Absorption Spectrophotometer (AAS) was used widely for specific determination of very low elemental concentrations in serum samples [18,19].

Hepatitis-C is becoming a major public health problem in Pakistan, with 4.5% population are infected with HCV [20]. In the present study, alterations in Fe levels in serum samples of female HCV patients at chronic stage were evaluated in order to detect the response of IFN-α therapy at different time intervals (24 and 48 weeks). For comparison purposes, blood samples of healthy age-matched females as referents were also analyzed.

Materials and Methods

Instrumentation

A Peel (Osaka, Japan) domestic microwave oven (maximum heating power of 900 W) was used for digestion of the serum samples. The analysis of element was carried out by means of Analyst 700, Perkin Elmer (Norwalk, CT, USA) atomic absorption spectrometer equipped with deuterium background correction and electrothermal atomizer. Single element hollow cathode lamp was operated at 7.5 mA with

analytical wavelengths at 248.5 nm. The flow rates of air (oxidant) and acetylene were 17.0 and 2.0 L/min, respectively. The other instrumental conditions were used according to the manufacturer's recommendation. Acid-washed Poly Tetra Fluoro Ethylene (PTFE) vessels and flasks were used for preparing and storing solutions.

Reagents and Standard Solutions

Ultrapure water obtained from ELGA Lab Water system ELGA (Bucks, UK) was used throughout the work. Concentrated HNO_3 (65%) and H_2O_2 (30%) obtained from Merck (Darmstadt, Germany) were checked for possible contamination of trace metals. Working standard solutions of Fe were prepared prior to their use by stepwise dilution of Certified standard solution (1,000 ppm) obtained from Fluke Karmic (Bucks, Switzerland), with 0.2 M HNO_3. All solutions were stored in PTFE bottles at 4°C. For the accuracy of methodology, Certified Reference Materials (CRMs) of Clinches control lyophilized® human serum Recipe (Munich, Germany) was used. All glassware and plastic materials used were previously soaked for 24 h in 2 M HNO_3, washed with distilled water, finally rinsed with Mille Q water, dried, and stored in class 100 laminar flow hoods.

Study subjects

One hundred sixty patients have Hepatitis C Virus (HCV) at chronic stage, with confirmed histopathological evidences were selected. The patients are attending the outpatient clinic and admitted in Hepatic-Gastroenterology Department of Hospital linked with Liaquat University of Medical and Health Sciences Jamshoro. The duration of diagnosed HCV disease is 2–5 years. Diagnosis of Hepatitis C Virus (HCV) infection was carried out by serum HCV RNA (Polymerase Chain Reaction; PCR), as well as the biochemical parameters such as serum Alanine Aminotransferase (ALT) level. The HCV genotyping were performed at the pathological laboratory of Liaqut Medical University. Among selected patients, 148 (92%) have HCV genotype 3, while 8% have other genotypes. Histological quantification of hepatic iron was not carried out in present study. For comparative purpose, 140 healthy subjects as referents (mostly the relatives of patients) of the same age group (30-50 years) were selected. They all patients and referents were residents of Hyderabad and different areas of Sindh, Pakistan. Patients were evaluated by laboratory tests and ultrasonography after full physical examination. At the start of the study, weight, height, blood pressure, and biochemical data of the participants were measured and recorded (Table 1).

Parameter	Control	Hepatitis C	After 6 months treatments	After 12 months treatments
BMI	25.3 ± 1.6	23.4 ± 2.9	23.6 ± 1.03	23.9 ± 1.25
Hb (13.2-17.3) g/dL	14.8 ± 1.5	11.2 ± 1.5	11.8 ± 0.92	12.6 ± 1.2
Htc (39-49) %	44.6 ± 3.1	30.9 ± 6.02	35.5 ± 1.56	41.7 ± 2.08
Bilirubin (3-20 µmoL/L)	14.6 ± 1.05	47.1 ± 2.08	34.8 ± 3.17	25.0 ± 2.55
Albumin (g/L) (40-52)	46.3 ± 2.55	25.8 ± 1.05	32.3± 2.08	36.1 ± 3.05
Urine Creatinine (27.0-260 mg/dL)	106 ± 14.9	270 ± 22.6	214± 17.5	171 ± 13.8
ALT (0-40 U/L)	26.2 ± 2.6	74.9 ± 10.5	55.2± 5.5	43.1 ± 6.42
AST (0-37 U/L)	14.3 ± 1.8	57.5 ± 6.32	45.5± 4.59	39.7 ± 4.98
ALP (37-147 U/L)	69.5 ± 3.1	180 ± 8.59	157 ± 10.5	137 ± 7.56
GGT (0-57 U/L)	28.3 ± 2.6	119 ± 11.8	98.2 ± 9.63	80.3 ± 8.05
SIBC (150-560 ng/dL)	236.8 ± 3.2	397 ± 35.6	316 ± 25.8	253 ± 19.6
TS (20-55 %)	25.4 ± 3.2	58.9 ± 7.3	51.1 ± 3.15	45.1 ± 2.08
Ferritin (28-80 ng/mL)	53.6 ± 3.4	252 ± 32.9	204 ± 20.6	136 ± 12.6
Total cholesterol (123-200 mg/dL)	136.5 ± 9.3	278.7 ± 26.3	236 ± 15.8	171 ± 19.5

Table 1: Clinical and biochemical characteristics of female HCV patients and referents after 6 and 12 months of IFN-α therapy. Values in parentheses, normal range. BMI: body mass index; Hb: Hemoglobin; Htc: Haematocrit; ALT: Alanine Minotransferase; ALP: Alkaline Phosphatase; GGT: Gamma Glutamyl Transferase; SIBC: Serum Iron Binding Capacity; TS: Transferrin Saturation.

The preliminary exclusion criteria for patients and referents were hypertension, alcoholism, diabetes, female subjects taking oral contraceptives, cardiovascular disease, and intake of any vitamin and minerals that could affect oxidative parameters. Among the selected HCV patients >35% of them were apparently worse in terms of chronic illnesses, malnutrition, poverty, and ignorance of disease for a long time. Histological evidences of HCV infections are not reported in present work.

The all possible sources of contamination in working laboratory were avoided. Written informed consent was obtained from all participants (Patients and referents). The study protocol and consent forms were approved by the Institutional Review Boards Sindh University Jamshoro, Pakistan. Among total patients 70% complete their six months treatment while only 42% have IFN-α therapy for one year. Although the government agencies provide IFN-α to people, but due to ignorance and sometime unavailability of medicine people does not continue the treatment for one year.

Protocol of therapy

The patients were treated with Interferon-α (Interon- Scherring, USA) alone. An injection of 3 million units of IFN-α was administered twice per week for 24 weeks (6 months) and 12 month. After treatment course for different time intervals (24 and 48 week), the virological exams and biochemical parameters were determined. Response to treatment was defined as serum virus less than 200,000 copy/mL (quantitative PCR) or a negative PCR (qualitative PCR). Blood serum samples were obtained for quantitative determination of Fe.

Blood sampling

The venous blood samples (5 mL) from patients and referents were collected after 12 h fasting, using metal free safety heparinized Vacutainer® blood collecting tubes (Becton Dickinson, Rutherford®, USA) between 9:30 and 11:00 am. About 2 mL of blood samples were sent to the pathological laboratories of hospital for biochemical tests using standard methods. Remaining (3 mL) samples were used for separating the sera. The blood is allowed to clot at room temperature for 15-30 min. When the blood sample was clotted completely, then it is centrifuged for 5-10 min at 2,500 rpm. The supernatant fluid was separated by a Pasteur pipette, labeled, and stored at -20°C until analysis.

Microwave-Assisted Acid Digestion

Duplicate of serum samples (0.5 mL) of each patient and referent whereas replicate six samples of certified material (serum) were directly taken into PTFE flasks. About 2 mL of a freshly prepared mixture of concentrated HNO_3-H_2O_2 (2:1, v/v) was added to each flask and kept for 10 min at room temperature, then the flasks were placed in covered PTFE container. The contents of flasks were heated following a one-stage digestion program at 80% of total power (900 W). Complete digestion of serum samples required 2-4 min. After the digestion, the flasks were left to cool, and the resulting solution was evaporated to semidried mass for the removal of excess acid. About 5 mL of 0.1 $MHNO_3$ were added to the contents of the flasks, shaken well, and filtered through a Whitman No. 42 filter paper, diluted with deionized water up to 10.0 mL in volumetric flasks. A blank extraction (without sample) was carried out through the procedure. Blanks and standard solutions were prepared in a similar acid matrix.

Statistical analysis

All statistical analyses were performed using the computer program Excel (Microsoft Corp., Redmond, WA, USA) and Minitab 13.2 (Minitab Inc., State College, PA, USA). The results of serum samples of referents and HCV patients before and after treatment (6 and 12 months) are reported as mean values with Standard Deviation (SD). The paired Student's t-test was used for comparison of biochemical and iron levels in serum samples of HCV patients before and after IFN therapy. The distribution of the data in each study group was checked by the Shapiro–Wilk test for normality. Nonparametric Mann-Whitney U tests were applied to test for significant differences in metal concentration between referents and patients. All relationships were significant at 95 % confidence interval ($p<0.05$), unless otherwise noted.

Analytical Figures of Merit

The calibration graph for Fe was linear with a correlation coefficient of >0.998 at the quantification limit up to 20 µg/L. The Limit of Detection (LOD) was defined as 3 s/m, where s is the standard deviation corresponding to ten blank injections and m is the slope of the calibration graph. The LOD is equal to 0.45 ng/g Fe. The validity and efficiency of the Microwave-assisted acid Digestion (MWD) method were checked with certified value of Clincheck control lyophilized® human serum Recipe (Munich, Germany), >98.0% recovery was obtained (Table 2). No significant differences was observed ($p>0.05$) when comparing the values obtained by MWD and certified value.

Certified value	Microwave assisted digested method	% Recovery	tabulated
1.3 ± 0.3	1.27 ± 0.10[a] (7.87)[b]	c97.7	0.819

Table 2: Validsation of methodology to determined iron certified reference material (mg L^{-1}). Key: [a]mean ± standard deviation, [b]Value in ( )=RSD, [c]% Recovery=(Obtained value by MWD)/(Certified value), [t]Critical=2.57.

Results

The resulted data indicates that the metabolism of Fe was altered, which may be a key factor in the pathogenesis of HCV before and after INF-α therapy. The mean concentrations with standard deviations of Fe in serum samples of referents, HCV patients before and after treatment with IFN-α therapy for 24 and 48 week are shown in Table 3. After treatment, HCV RNA level was undetectable in 32% and 58% patients, for 24 and 48 weeks, respectively.

	Control	Before treatment	After treatment		P value
			6 months	12 months	<0.01-0.001
Female	1.08 ± 0.14a	3.42 ± 0.39	2.39 ± 0.28	1.56 ± 0.27	

Table 3: Concentration of Fe (mg L^{-1}) in serum samples of female HCV patients before and after IFN-α therapy. A mean ± standard deviation.

The resulted data indicated that the level of Fe were significantly higher in serum samples of HCV patients as compared to healthy referents ($p<0.01$). The levels of Fe in serum samples of HCV patients were found as median at 95% confidence interval 3.3 [3.34-3.50], which was significantly reduced after treatment with IFN-α for 24 and 48 week, 2.3 [2.34-2.45] and 1.5 [1.50-1.61], ($p<0.01$), respectively. The level of Fe in serum samples after 48 week treatment was significantly reduced but still 38% higher than referents. The distribution of Fe resulted data in serum samples of referents and patients was checked by the Shapiro–Wilk test for normality. It was observed that there is no significant difference in Fe level between normal and log normal distribution. So for comparative purpose, we use data of Fe in referents and patients before and after therapy at normal distributions.

The biochemical parameters of patients (before and after therapy at different time intervals) are shown in Table 1. After therapy, the improvement in different biochemical parameters of HCV patients were observed as compared to the values obtained before any treatment. The per cent of hemoglobin in HCV patients was enhanced up to 1-2% after treatment. After therapy, the ferritin levels were decreased in the range of 19-46 %. The albumin level was enhanced in the range of 20.2-28.6%, after therapy in HCV patients, indicating the improvement in liver functions (Table 1). ALT levels in serum of HCV infected patients before therapy was significant increased compared to control group; and after treatment at different time intervals there was significant decrease in its levels 26.4-42.5% when compared to those values obtained before treatment, but still high than the control group. Statistically significant positive correlation between ALT and Fe was found after treatment [$r=0.42$, $p<0.05$].

Discussion

The changes in the hematological and biochemical parameters in HCV female patients before and after IFN-α therapy at different time intervals in relation to healthy females of the same age group are shown in Table 1. A hematological and biochemical parameters, including Hemoglobin (Hb), albumin, globulin, total bilirubin, aspartate aminotransferase, and alanine aminotransferase, are monitored routinely for the diagnosis of hepatic diseases. Serum Fe Binding Capacity (SIBC) and ferritin levels are the principal tests used in the evaluation of Fe burden [21]. The level of bilirubin was found to be higher in HCV patients as compared to referents; our results are consisted with literature statement that the high bilirubin level reflects liver cell damage or bile duct damage [22].

The significant increases in the levels of ferritin and bilirubin ($p<0.05$), while decreases in the concentrations of Hb and albumin in the blood of HCV patients were observed with related to referents. After therapy, the enhancement in Hb and albumin levels while lowering the ferritin and serum bilirubin indicates the improvement in liver functions. The progress in liver function was higher in the case of 48 weeks therapy, which is confirmed by the 58% of HCV patients having a negative HCV RNA test.

The Fe was first suggested as potentially important in the pathogenesis of HCV infection in 1992, with the demonstration of elevated Fe indices in many patients with chronic hepatitis C [23]. Normal Fe metabolism in the liver may be disrupted by viral hepatitis, and the accumulation of Fe in the liver is increased [24].

Increased hepatic Fe had the greatest association with the severity of liver fibrosis [25]. The prevalence of hemochromatosis gene mutations associated with hereditary hemochromatosis is increased among Asian subjects with hepatitis [26]. Fe could potentially play a supporting role in the lipid peroxidation and fibrogenesis leading to and progression of hepatitis [27]. It is known that Fe^{+2} in the presence of hydrogen peroxide generate hydroxyl radicals through the Fenton reaction [28]. It is plausible that ROS production during chronic HCV infection is due to high Fe level in hepatic tissue where free Fe is known to be a potent catalyst for the production of free radicals [29]. Hepatic Fe deposits have been associated with the degree of liver inflammation and damage in HCV-infected liver tissues. However, the mechanisms involved in the process of Fe deposition need to be better clarified [30]. Fe may worsen the clinical course of HCV infection by causing oxidant stress in non-parenchymal cells, which appears to cause irreversible mitochondrial derangement associated with the onset of hepatic fibrosis [31,32].

In the present study the changes in iron absorption from gastrointestinal tract was not evaluate that this may have contributed to hepatic iron accumulation following INF-therapy. The results of the present study showed that, the Fe levels in serum of HCV infected patients before and after treatment were significantly altered when compared to referent group. These results were in agreement with other study who reported that, there is an obvious increase of Fe contents in the serum of HCV patients [33-55].

Conclusion

Present results suggested that liver functional impairment may alter the metabolism of Fe, Our findings imply that the levels of Fe in serum samples might serve as biochemical parameters with a predictive value for the responsiveness of patients to interferon therapy.

In most of the cases people start treatment in late stages of HCV, because this viral disease is asymptomatic, while low literacy rate, unhygienic conditions and poverty, especially among rural population of Pakistan, are also common. Mostly peoples are treated with alone INF-α therapy. The level of Fe in serum samples were significantly reduced after longer period of treatment with INF-α. However, large, randomized, controlled trials using Fe reduction or longer duration of INF-α therapy will be needed. Often the Hepatitis C relapses, so the long-term use of INF-α (for more than 1 year) may perhaps be appropriate to suppress disease activity.

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