Pharmaceutica Analytica Acta

Hassan etal., Pharm Anal Acta 2016, 7:7
DOI: 10.4172/2153-2435.1000500

Research Article Open Access

Estimate the Levels of Copper in Serum Samples of Hepatitis C Patients at Different Stages after Interferon- α Therapy

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Received date: June 28, 2016; **Accepted date:** Jul 29, 2016; **Published date:** Jul 31, 2016

Abstract

In this study, we evaluated the copper (Cu) levels in serum samples of patients infected by hepatitis C virus (HCV) at different stages and examined the changes in its content before and after interferon α (IFN-α) therapy with related to healthy subjects. The 199 HCV patients of both genders at different stages including (acute, fibrosis and cirrhosis) were enrolled in this study, who have completed IFN-α treatment and were followed for at least 1 year after the end the therapy. We have also evaluated the changes in biochemical parameters before and after treatment. Blood samples were collected from patients before and after treatment of IFN-α therapy. For comparative purposes healthy subjects of same age group (30-50) were also selected. The trace level of Cu in serum samples was determined by flame atomic absorption spectrometry after preconcentration by dual-cloud point extraction method. It was observed that after INF-α treatment the level of Cu in serum samples of HCV patients were reduced in decreasing order such as acute> fibrosis> cirrhosis.

Keywords: Copper; Hepatitis C; Interferon therapy; Microwave assisted acid digestion; Flame atomic absorption spectrometry

Introduction

Hepatitis is the most common cause of chronic liver diseases around the world, and according to WHO reports, more than 170 million persons are suffering by hepatitis C [1,2]. HCV infection frequently leads to chronic hepatitis with increasing risk of developing liver cirrhosis and hepatocellular carcinoma (HCC). Interferon with or without other drugs, is the only medicine with proven efficacy in treating chronic HCV infections. Unfortunately, these therapeutic models maintain the rate of sustained virology response to approximately 10-40% [3-5]. Copper (Cu) is an essential trace element which participates in many enzymatic and redox reactions. The Cu is associated with a number of metalloproteins [6,7]. The major functions of Cu metalloproteins are oxidation-reduction reactions as Cu-containing enzymes bind and react directly with molecular oxygen. During infections or inflammatory stress, serum Cu concentration increases due to acute-phase activity of interleukin-1 [8,9]. Exposure to high levels of Cu results in various changes in the liver tissues that are consistent with oxidative damage to membranes and molecules. Lipid peroxidation causes dysfunction in the cell membrane, decreased fluidity, inactivation of enzymes and changes ion permeability [10,11]. Hatano studied that Cu accumulation in fibrotic livers caused by chronic hepatitis C may contribute to hepatic injury [12]. Reactive Cu can participate in liver damage directly or indirectly through Kupfer cell's stimulation [13]. Scientists agree that the toxic effects of Cu are related to oxidative stress [14,15]. It was reported that Cu concentration in biological samples varies with different types of liver diseases because this element may have a direct hepatic toxicity or can be enhanced the consequence of the impaired liver function [16,17]. The Cu acts as a cofactor against hepatic fibrosis in chronic liver diseases, particularly in the biosynthesis of collagen. As the disease progresses from chronic hepatitis to liver cirrhosis, Cu levels increase in biological fluids [18].

In view of the above facts it is necessary to evaluate the alteration in Cu levels of biological samples of HCV patients with related to non-diseased subjects. Flame atomic absorption spectrometry is by far the most widely employed technique for analyte determination [19,20], but it is not sufficiently sensitive for the direct determination of trace level of Cu in biological/environmental samples, mostly due to the complexity of the matrix and the low concentration, so it needs prior separation and preconcentration [21,22]. The analytical potential of an enrich method, cloud point extraction (CPE) has been discussed by several authors, comprising this method as green chemistry [23,24]. The CPE is based on the phase behavior of non-ionic surfactants in aqueous solutions, which exhibit phase separation after an increase in temperature or the addition of a chelating agent [25]. The drawbacks of traditional CPE problems are overcome by dual-cloud point extraction (d-CPE) method [26]. Due to the introduction of the second CPE phase, the effects of surfactant on the analysis and separation of metals are eliminated. The removal of interfering species through the d-CPE procedure improves the efficiency and selectivity of proposed method, the back extraction of analyte in the aqueous sample solution is naturally compatible with the conditions of flame atomic absorption spectrometry [27,28]. The aim of present study was to determine the levels of Cu in serum samples of HCV patients of both genders at different stages (acute, fibrosis and cirrhosis also referred as 1st, 2nd and 3rd stages of HCV), before and after INF-α therapy. To determine trace levels of Cu in serum samples an innovative preconcentration method was applied for enrichment, before analysis by flame atomic absorption spectrometry.

Materials and Methods

Instrumentation

A pH meter (Ecoscan Ion 6, Malaysia) was employed for pH adjustments. A PEL domestic microwave oven (Osaka, Japan), programmable for time and microwave power from 100 to 900 W, was used for digestion of serum samples. Centrifugation was carried out using a WIROWKA Laboratory jna type WE-1, nr-6933 centrifuge (speed range 0-6000 rpm, timer 0-60 min, 220/50 Hz, Mechanika Phecyzyjna, Poland). A Perkin–Elmer Model AAnalyst 700 (Norwalk, CT) flame atomic absorption spectrophotometer was used. The hollow cathode lamp of Cu was run under the conditions suggested by the manufacturer. A single element hollow cathode lamp was operated at 7.0 mA and spectral bandwidth of 0.7 nm. The analytical wavelength was set at 324.8 nm. The acetylene flow rate and the burner height were adjusted in order to obtain the maximum absorbance signal.

Chemical reagents and glassware

Ultrapure water obtained from a ELGA lab water system (Bucks, UK) was used throughout the study. Concentrated nitric acid (65%) and hydrogen peroxide (30%), hydrochloric acid (37%) were obtained from Merck (Darmstadt, Germany). Working standard solutions of Cu were prepared immediately before their use, by stepwise dilution of certified standard solution (1000 mg L^{-1}) obtained from Fluka Kamica (Buchs, Switzerland), with 0.2 mol L^{-1} HNO_3. The 1-(2-pyridylazo)-2-naphthol (PAN), were obtained from (Fluka) and prepared by dissolving appropriate amount of these reagents in 10 mL ethanol (Merck) and diluting to 100 mL with 0.01 mol L^{-1} acetic acid. The nonionic surfactant Triton X-114 was obtained from Sigma (St. Louis, MO, USA) and used without further purification. A 1% (v/v) nonionic surfactant solution was prepared by dissolving 2 mL of Triton X-114 (Merck) in 100 mL distilled water. A stock buffer solution was prepared by dissolving appropriate amounts of acetic acid and its sodium salt in ultrapure water, and pH was adjusted with 0.1 mol mol/L HCI/NaOH. For the accuracy of methodology, certified reference materials (CRMs) of Clincheck control lyophilized® human serum Recipe (Munich, Germany) was used. All materials and glassware used for Cu analyses, were kept in 10% HNO_3 for at least 24 h and subsequently rinsed four times in ultrapure water to avoid contamination.

Study subjects

For present study, the patients attending outpatient clinic and admitted in hepato-gastroenterology ward of civil hospital of Hyderabad were selected. Blood samples were collected from 199 hepatitis C (HCV) patients, tested by anti-HCV antibodies test (positive RNA test/ PCR test). The HCV patients were grouped as 41 males and 36 females at first stage of HCV (after diagnosis, termed as acute phase), 35 males and 31 females at second stage/fibrosis (after more than 1 year, ultrasonic test), while 29 males and 27 females at last stage of HCV/cirrhosis (ultrasound test). For comparative purpose, 143 healthy subjects (69 males and 74 females) as referents (mostly the relatives of patients) of same age group (30-50 years) were selected. They all were residents of Hyderabad, Mirpur khas and from different areas of Sindh, Pakistan. Among these patients, those who received antiviral treatment with interferon alone were included in the present study after taking their written informed consent. 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). All patients and referents provided a written consent, confirming that they accepted conditions of giving blood samples and were informed about the whole experimental procedures. A questionnaire was administered to all patients and referents to collect details of their physical data, ethnic origin, dietary habits, age, and consent. The criteria for the selection of referent subjects were based on same age group, socioeconomic status, residential areas, and dietary habits. The preliminary exclusion criteria for patients and referents were hypertension, alcoholism, diabetes, cardiovascular disease, and intake of any vitamin and minerals that could affect oxidative parameters. Among HCV patients <50%, especially those have liver cirrhotic, the health condition was apparently worse in terms of chronic illnesses, malnutrition, poverty, and ignorance of disease for a long time. During this study intervals, 3 male (8%) and 6 females (14%) at acute phase, 5 (16%) male and 4 female (11%), while at cirrhotic stage 4 male (15%) and 5 female (17%), discontinue the interferon treatment. The care was taken to avoid all possible sources of contamination in working laboratory. The study protocol and consent forms were approved by the Institutional Review Board, University of Sindh, Jamshoro, Pakistan.

Blood sampling

The venous blood samples (5 mL) from patients and referents were collected after 12 h fasting, using metal free safety heparinzed Vacutainer® blood collecting tubes (Becton Dickinson, Rutherford®, USA), between 9:30 and 11:00 a.m. 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. 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.

Sample preparation and preconcentration

The samples were digested using microwave assisted digestion method. For this purpose, duplicate serum samples (0.5 mL) of each patient and referent subject and replicate six samples of each certified materials (serum) were directly placed 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 mol/L of HNO_3 was added to the contents of the flasks, shaken well, and filtered through a Whatman No. 42 filter paper and diluted with deionized water up to 10.0 mL in volumetric flasks [29,30]. Then the samples were preconcentrated using d-CPE method as reported in our previous work [29]. Brief summary is given below.

Procedure for d-CPE

The procedure of d-CPE was based on two steps of the conventional cloud point extraction process. For the first CPE stage, aliquots of 10 mL of a standard solution containing Cu ions in the range of 10-50 µg/ Land duplicate samples of acid digested serum samples of each patient

were transferred into centrifuge tubes with glass stopper (25 mL in capacity). Added 0.5 mL of (PAN) (4.0×10^{-4} mol/L), 2 mL of Triton X-114 (0.15%), v/v) and 2 mL of different buffers to adjust a pH 7 with 0.1 mol/L of HNO_3/NaOH. The contents of tubes were kept in a thermostatic bath at 45°C for 10 min. Separation of the phases was achieved by centrifugation for 10 min at 2500 rpm, further increase of time has no significant effects on the extraction efficiency of studied element. The tubes were placed in an ice bath to increase the viscosity of the surfactant-rich phase. Then, the aqueous phase was decanted. Instead of addition of diluents or analysis, the surfactant-rich phase containing the complexes was treated with 2 mL of 0.3 mol/L of HNO_3 and placed in thermostatic bath at 45°C for 10 min as the second round of cloud point extraction. After different time interval the content of tubes were centrifuged for 10 min at 2500 rpm. The supernatant obtained after d-CPE, was introduced into FAAS.

Parameters	Male				Female			
	Referents (n=69)	HCV Patients			Referents (n=74)	HCV Patients		
		1st stage (n=41)	2nd stage (n=35)	3rd stage (n=29)	1st stage (n=36)	2nd stage (n=31)	3rd stage (n=27)	
BMI (g/dL)								
Before treatment	26.8 ± 1.55	26.3 ± 1.41	25.4 ± 1.37	25.8± 1.27	26.2 ± 1.76	25.9 ± 1.62	25.6 ± 2.08	25.3 ± 1.92
After treatment		26.6 ± 1.07	25.7 ± 1.38	26.1± 1.57		26.4 ± 1.54	26.0 ± 1.27	25.7 ± 1.64
Hb (13.2-17.3 %)								
Before treatment	16.5 ± 1.22	14.6 ± 1.25	13.7 ± 1.15	12.6± 1.36	15.9 ± 1.39	14.6 ± 1.17	13.3 ± 0.49	12.3 ± 0.79
After treatment		15.0 ± 1.54	14.1 ± 1.08	12.96 ± 1.44		15.16 ± 1.03	13.86 ± 1.54	12.76 ± 1.51
Htc (39-49)								
Before treatment	45.8 ± 2.25	31.7 ± 2.48	27.9 ± 1.96	25.8 ± 3.72	44.7 ± 2.19	32.5 ± 3.59	28.7 ± 4.06	23.9 ± 2.06
After treatment		38.6 ± 1.21	33.7 ± 1.63	29.6± 1.74		38.8 ± 1.62	32.3 ± 1.98	26.7 ± 1.70
Bilirubin (µmol/L) (3-20)								
Before treatment	13.7 ± 3.26	36.7 ± 2.95	46.2 ± 3.94	56.9 ± 1.95	14.2 ± 2.59	37.9 ± 2.06	47.1 ± 2.28	57.3 ± 1.69
After treatment		13.8 ± 5.08	27.6 ± 7.25	36.8± 4.24		16.6 ± 4.23	31.9 ± 2.67	38.2 ± 6.45
Albumin (g/L) (40-52)								
Before treatment	46.3 ± 3.63	31.9 ± 1.85	28.9 ± 3.52	25.7 ± 2.43	45.8 ± 2.61	29.6 ± 1.45	28.2 ± 1.73	24.7 ± 1.93
After treatment		39.1 ± 4.37	34.5 ± 6.02	29.7± 4.39		35.7± 3.27	33.4 ± 3.98	28.0 ± 4.45
ALT (0-40 U/L)								
Before treatment	27.4 ± 1.63	59.3 ± 7.93	75.8 ± 5.92	98.5 ± 3.27	26.9 ± 1.31	60.5 ± 2.68	74.8 ± 4.92	95.2 ± 4.25
After treatment		40.0 ± 4.25	54.9 ± 6.34	76.3 ± 5.03		42.0 ± 5.24	55.7 ± 4.15	76.7 ± 7.05
AST (0-37 U/L)								
Before treatment	17.9 ± 1.99	42.5 ± 9.86	54.9 ± 16.8	139 ± 12.9	18.7 ± 2.48	44.1 ± 7.92	56.2 ± 10.2	135 ± 9.95
After treatment		35.1 ± 5.07	47.5 ± 7.15	123.0 ± 9.36		35.9± 6.20	48.0 ± 5.16	119.6 ± 10.6
ALP (37-147 U/L)								
Before treatment	65.6 ± 2.92	158 ± 9.51	175 ± 7.52	285 ± 9.15	67.3 ± 8.59	162 ± 7.59	179 ± 6.17	285 ± 6.28
After treatment		114 ± 9.05	141 ± 12.5	246 ± 16.8		114 ± 10.5	143 ± 8.65	241 ± 15.2
GGT (0-57 U/L)								
Before treatment	29.6 ± 4.28	79.3 ± 4.58	126 ± 8.58	159 ± 10.5	31.5 ± 3.56	72.6 ± 3.64	116 ± 4.19	154 ± 5.62
After treatment		59.1 ± 6.52	103 ± 10.5	137 ± 8.27		53.2 ± 7.24	94.9 ± 10.5	131 ± 12.5
SIBC (150-560 ng/dL)								

Before treatment	229 ± 6.37	310 ± 5.10	390 ± 8.35	545 ± 12.3	236 ± 8.51	307 ± 5.69	386 ± 7.15	553 ± 10.8
After treatment		220 ± 8.05	302 ±7.65	438 ±15.3		223 ± 16.3	300 ± 8.02	454 ± 5.2
TS (20-55 %)								
Before treatment	27.3 ± 4.29	56.8 ± 3.28	72.5 ± 2.73	84.9 ± 3.22	26.4 ± 2.89	51.2 ± 3.51	68.5 ± 3.65	82.6 ± 3.49
After treatment		43.2 ± 5.49	58.3 ± 7.25	70.7 ± 9.36		38.7 ± 6.09	54.7 ± 7.25	67.5 ± 8.32
Ferritin (28-80 ng/mL)								
Before treatment	54.7 ± 2.17	152 ± 5.28	252 ± 3.64	359 ± 5.19	56.5 ± 4.75	149 ± 8.19	239 ± 5.62	345 ± 6.72
After treatment		103 ± 12.7	190 ± 15.2	293 ± 20.5		97.6 ± 10.3	179 ± 8.05	285 ± 15.4

Table 1: Clinical and biochemical characteristics of HCV patients and referents.

Statistical analysis

The different software computer program (Excel and Minitab 13.2) was used for calculation of resulted data. The obtained results of Cu at different stages of HCV before and after INF therapy were calculated as mean ± standard deviation. The resulted data of each HCV patients group were checked by the Shapiro–Wilk test for normality. The difference between referents and patients was calculated by Nonparametric Mann-Whitney U tests.

Analytical characteristics of method

The calibration graph of Cu was linear with a correlation coefficient (R2) of 0.992 in the concentration range of (10-50 µg/L) and subjecting to preconcentration method, d-CPE. The analytical characteristic of d-CPE is shown in Table 2, whereas detail is present in our previous work [29].

The precision of the method, calculated as the relative standard deviation of six independent measurements carried out on a standard solution of Cu (20.0 µg/L) The limit of detection is defined as the concentration equivalent to three times the standard deviation of six measurements of the blank, after preconcentration was 0.047 µg/L.

The validity and accuracy of proposed method were checked by analysis of Cu in certified sample of serum (CRM). The obtained % recovery was <98% of the certified value of Cu in CRM.

Concentration range of Cu (µg/L)	10-100
Limit of detection[a] (LOD)	0.047
R2 (correlation coefficient)	0.992
Repeatability (RSD%)[b] (n=6)	>5.0
Enrichment factor[c]	78
Preconcentration factor[d]	30

LOD=was calculated as 3.3 a/S Where, S=Slope,

[a]Standard deviation, corresponding to ten blank injections.

[b]Cu concentration was 20 µg/L for which the % relative standard deviation was obtained.

[c]Calculated as the ratio of slope of preconcentrated samples to that obtained without preconcentration.

[d]Calculated as the ratio of volume before and after preconcentration.

Table 2: Analytical characteristic of the preconcentration method dual-cloud point extraction for copper.

Results and Discussions

Optimization of d-CPE procedure

Six factors, such as pH, concentration of complexing agent (PAN), surfactant concentration (%), incubation time, concentration of nitric acid for extraction of analyte at the second step of CPE and temperature were selected to point out the competence of the proposed method.

The detail about optimization and analytical characteristics are mentioned in our previous work [29].

	HCV Patients			Referents
	Acute	Fibrosis	Cirrhosis	
Female				
Before treatment	2.58 ± 0.35	3.77 ± 0.13	5.15 ± 0.57	1.17 ± 0.15
After treatment	1.48 ± 0.20	2.26 ± 0.08	4.25 ± 0.47	
Male				
Before treatment	2.45 ± 0.38	3.63 ± 0.28	4.93 ± 0.60	1.11 ± 0.14
After treatment	1.41 ± 0.22	2.18 ± 0.17	4.07 ± 0.50	

Table 3: Concentration of copper in serum samples of various stages of HCV patients before and after interferon-α therapy.

The mean concentrations with standard deviations of Cu in serum samples before and after treatment with INF-therapy are shown in Table 3. The distribution of resulted data of Cu in serum samples of HCV patients before and after treatment was checked by the Shapiro-Wilk's test for normality. No significant difference was observed between normal and lognormal distribution of Cu values in serum samples of patients and controls. So for comparative purpose we use data of Cu in referents and patients of both genders at normal

distributions. The results indicated higher level of Cu in serum samples of male HCV patients at acute, fibrosis and cirrhosis stages before treatment at 95% confidence intervals with median values as, 2.58 [CI: 2.18, 2.68], 3.63 [CI: 3.46, 3.85], 4.83[CI: 4.53, 5.18], µg/L, respectively. After treatment the levels of Cu was reduced in HCV patients at different stages, acute, fibrosis and cirrhosis, 1.48 [CI: 1.25-1.54], 2.17 [CI: 2.07-2.31], 3.99 [CI: 3.74-4.27] µg/L, respectively. It was observed that significant effect of INF-α therapy was observed in HCV patient at acute stage ($p<0.01$), but the obtained values of Cu were still 10-13% higher than healthy referents results, 1.12 [CI: 1.09-1.16] µg/L. Same trend was observed in female HCV patients at different stages. The effects of INF-α therapy was more effective at 1st stage of HCV infection (acute), where the levels of Cu reduced in the range of 40-50% in serum samples after one year complete treatment. It was observed that at cirrhotic stage the INF-α is not so effective, where only 14-18% of Cu levels in serum samples were decreased.

The biochemical parameters of patients (before and after therapy) 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.

After therapy the improvement in biochemical parameter were observed in HCV patients at different stages in decreasing orders, as acute>fibrosis>cirrhosis. The % of hemoglobin in HCV patients was enhanced in the range of 2.4-3.7% after treatment. After therapy, the ferritin levels were decreased in the range of 17.4-34.5%. The albumin levels were enhanced in the range of 13.3-22.6%, after therapy in HCV patients at different stages, indicating the improvement in liver functions (Table 1). ALT levels in serum of HCV infected patients before treatment was significant increased compared to control group; and after treatment there was reduced in the range of 19.4-32.5%, when compared to those values obtained before treatment, but still higher than control group. Statistically significant positive correlation between ALT and Cu was observed after treatment [$r=0.432-0.487$, $p<0.05$].

The Cu is an essential element for the production of hemoglobin. It is also involved in the metabolism of collagen, the protein responsible for the integrity of bone, cartilage, skin and tendon, and elastin, major component of large blood vessels [30,31]. But in excess, Cu is highly toxic, and results in cellular damage and hepatocellular carcinoma [32]. In the presence of available cellular reductants, Cu compounds in low molecular weight forms may play a catalytic role in the initiation of free radical reactions. The resulting oxyradicals have the potential to damage cellular lipids, nucleic acids, proteins, and carbohydrates, resulting in wide-ranging impairment in cellular function and integrity [33]. The Cu in serum plays an important role in the interrelation among immunoglobulins (IgA, IgM, and IgG) in various liver diseases [12]. The accumulation of Cu in fibrotic livers caused by chronic HCV infection may contribute to hepatic injury.

Kalkan et al. [32] have been reported that the Cu levels were elevated in sera and have also suggested that this may probably reduce the defense strategies of the organism. Elevated serum Cu concentrations are seen in portal cirrhosis and hepatitis due to excessive Cu, which is normally excreted in the bile and is probably retained in the circulation [34,**35**]. In the liver, much of the Cu is bound with metallothionein, which may contribute to Cu overload in the liver [**36**]. Hayashi et al. suggested that reactive oxygen species produced by Cu accumulation induce several types of DNA damage, such as base alteration and DNA strand breaks in Long–Evans cinnamon rats. In this situation, breaks in DNA strands may not be repaired efficiently, and these unrepaired breaks may cause massive cell death leading to severe hepatic injury [37-39]. Versieck et al. [36] determined Cu concentration in serum during acute viral hepatitis, indicated that the mean serum Cu is significantly increased ($p<0.01$) in hepatitis patients. This elevation may be caused from necrosis of liver parenchyma, leading to an important release of hepatic Cu or decreased hepatobiliary excretions where it is principally excreted into the bile. Mine reported lower Cu content in the sera of chronic active hepatitis and cirrhosis patients. Their results also demonstrated that compared with the control group the sera of acute hepatitis and chronic hepatitis has the low level of Cu concentration. Kop reported the increase of Cu in serum with acute and chronic hepatitis because of increasing synthesis of indigo Cu protein. The accumulation of Cu in fibrotic livers caused by chronic hepatitis C may contribute to hepatic injury. Although the real mechanism is still unclear, excessive amounts of Cu may damage liver by oxidative stress [12].

Conclusion

The resulted data of present study demonstrated that the impairment of liver function may alter the mineral metabolism, in particular Cu in blood serum. Its determination in biological fluids is one of the good markers for diagnosing of hepatitis patients infected with HCV. Hepatitis virus activities were influenced by the increased of Cu in serum samples of HCV patients, whereas difference was apparent at cirrhotic stage. The real mechanism is not known at present, but excess Cu may damage the liver by oxidative stress. As the severity of HCV was increased, the improvements after INF-α therapy in biochemical parameters were reduced as compared to HCV infection at 1st stage. The level of Cu in serum samples of HCV patients was reduced after therapy but did not show statistical alterations at later stage as compared to those of healthy control subjects ($p<0.05$). Because, in most of the cases people start treatment in late stages of HCV, as this viral disease is asymptomatic. In study area, most of the HCV patients are treated with alone INF-α therapy, which is also safe and potentially promising means of treating patients with HCV, whereas it is more effective at initial stage of disease.

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