BBA Clinical 2 (2014) 72–78

Contents lists available at ScienceDirect

BBA Clinical

journal homepage: http://www.journals.elsevier.com/bba-clinical/

Viral load is associated with abnormal serum levels of micronutrients and glutathione and glutathione-dependent enzymes in genotype 3 HCV patients

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ARTICLE INFO

Article history:
Received 25 June 2014
Received in revised form 12 September 2014
Accepted 24 September 2014
Available online 2 October 2014

Keywords:
Hepatitis C viral load
Glutathione
Glutathione reductase
Glutathione peroxidase
Micronutrients

ABSTRACT

Background: Oxidative stress in hepatitis C patients has been linked to hepatitis C virus. We verified this assumption in HCV genotype 3 patients by detecting the relationship between viral load and certain specific oxidative stress markers like Cu, Mn, Fe, Se, Zn and glutathione and glutathione-dependent enzymes.
Method: Subjects (n = 200, average age 24 years) with quantitative HCV RNA polymerase chain reaction-proven genotype 3 hepatitis C were simultaneously evaluated. Cu, Mn, Fe, Se and Zn serum levels were by using atomic absorption spectrophotometer. Internationally accepted methods were used for viral load quantification of glutathione, GR and Gpx serum levels.
Result: There was a significant correlation between HCV viral load and studied parameters. With the increase of viral load from mild group (200,000–1,000,000 copies/ml) to severe group (5,000,000–25,000,000 copies/ml) the serum levels of Cu, Mn, Zn, and Fe and glutathione reductase were found to be abnormally high. However, in severe viral load group serum concentration of Se and glutathione was less than the healthy controls.
Conclusion: As a significant correlation was detected between the study parameters in genotype 3 HCV patients, it is concluded that the studied micronutrients and glutathione and glutathione-dependent enzymes are the biomolecular targets of HCV to induce oxidative stress.
General significance: Constant monitoring and regulation of the recommended biomolecular targets of HCV can improve the plight of more than 170 million patients suffering from hepatitis C virus around the globe.

1. Introduction

Worldwide 170 million individuals are estimated to be infected with hepatitis C virus [1]. It is one of the four most prevalent health problems globally and it leads to liver injury, cirrhosis, hepatocellular carcinoma and eventually to the death of affected patients. Hepatitis C virus is thought to be the second most common cause of hepatitis worldwide. The prevalence of HCV infection varies throughout the world. Unlike the developed country as the United States, the frequent source of transmission of HCV in developing countries is exposure to infected blood in healthcare and community settings [2–4].

In hepatitis C the patient faces different stages of infection. Acute hepatitis C is an initial stage marked by the appearance of HCV RNA in serum within 1 to 2 weeks of exposure and it is also accompanied by elevation in serum alanine aminotransferase (ALT) levels, and then jaundice. Antibody to HCV (anti-HCV) tends to appear late. Acute hepatitis is curable by medication. However, 55% to 85% of patients do not clear virus, and develop chronic hepatitis C. The chronic progression of hepatitis C involves progressive hepatic fibrosis, cirrhosis, and hepatocellular carcinoma [5].

Moreover, the entry of HCV virus in the cell is mediated by several receptors and co-receptors like CD81, occludin, claudin-1 and scavenger receptor class B type I [6]. The complex of enveloped nuclear capsid, apo E and VLDL act as LDL receptor ligand and binds to the surface receptor and enters the hepatocyte by internalization [7]. An internal ribosomal entry site situated inside the 5′ UTR is utilized for the translation of positive-sense HCV RNA [8].

After the entry of virus in the body the process of HCV replication starts and all the structural and non-structural components of HCV genome are associated with its replication. The length of HCV genome is 9.6 kb and it consists of three main regions. These are structural region with three genes (C, E1, E2), nonstructural region (p7, NS2, NS3, NS4A/B, NS5A/B) and the protein coding regions, flanked by UTRs at 5′ and 3′. The structural genes code for the core protein which makes the viral capsid and envelope glycoprotein. Nonstructural genes (NS2, NS3, NS4A/B, NS5A/B) and p7, an essential membrane protein that works as an ion channel, are involved in viral replication, poly-protein processing, and/or morphogenesis and viral release. The 5′ and 3′ UTRs are necessary for the expression and

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http://dx.doi.org/10.1016/j.bbacli.2014.09.006

replication of viral gene. Hepatitis C virus (HCV) has a poly (U) stretch tail of 98-nucleotide sequence downstream at the 3′ terminus of the genome. The 3′ tail of HCV with the nucleotide sequence is highly conserved. This highly conserved tail is present in whole HCV genome. This 3′ tail is very crucial for the initiation of replication of genome and the whole process of HCV replication is only dependent on the HCV genome [9–11].

However, according to some recent studies the process of HCV replication is affected by external factors like reactive oxygen species at a subcellular level. In hepatitis C patients, a state of oxidative stress is acquired by the body cell. In this process the equilibrium between the oxidant and antioxidant production is lost in the body and it leads to overproduction of reactive oxygen species [12]. Overproduction of ROS causes oxidative stress resulting in cellular damage which leads to a more free radical production [13]. In HCV patients ROS mainly originated from liver Kupffer and inflammatory cells producing weak immune response [14,15]. HCV core protein and NS proteins are reported to increase the production of ROS in the body and these proteins also interferes with normal working of cells by inhibiting electron transport chain, altering apoptosis, transcription process and cell signaling [16]. These malicious proteins further alter the level of endogenous antioxidants and anti-oxidant enzymes in the body [17]. NS proteins, particularly NS5A and NS3 are the major replication complexes and the ROS that are generated as a result of hepatitis C infection also attacks on these active HCV replication complexes and reduces the amount of NS3 and NS5A which results in the suppression of HCV RNA replication in human hepatoma cells [18]. So HCV replication is not benefited by the overproduction of ROS.

Along with signal transduction, HCV RNA replication suppression and gene regulation free radicals also play a role as toxins in the human body [19]. After exposure to HCV, the diseased liver produces excessive amount ROS from internal sources like mitochondria, inflammatory cells and peroxisomes [20]. In liver disease, the main sources responsible for the overproduction of ROS are endogenous sources such as the mitochondrial factor, peroxisomes, and activated inflammatory cells. These radicals damage cells by attacking vital cell components, such as the fat and protein constituents of the cell wall and the cell's genetic material. For instance, oxidative stress can induce enhanced metabolism of fat molecules (i.e., lipid peroxidation) that may generate biologically active molecules. Some of these molecules sometimes contribute to the development of fibrosis. However, the formation of oxygen radicals is a natural process and this occurs during numerous metabolic processes [21].

ROS acts as potential carcinogens that facilitate mutagenesis, tumor progression and tumor promotion, by having an effect on the redox-responsive signaling cascades. Therefore, enhanced ROS generation by HCV virus infection can cause enhanced oxidative stress that leads to activation of oncogenic transcription factors that ultimately ends at hepatocellular carcinoma. Two Transcription Factors, NF-κB (nuclear factor κ B) and AP-1 (Activator protein 1), are activated as a result of oxidative stress to the cells. Also, the two distinct protein Families namely, i) MAPK (Mitogen Activated Protein Kinases) and ii) The Redox Sensitive Kinases regulates the signal transduction pathways activated by ROS. Mitochondrial ROS are also generated by HCV RNA genome. The HCV non-structural protein 5A (NS5A) is associated with endoplasmic reticulum (ER)-nucleus signal transduction pathway. Endoplasmic reticulum stress is caused by the expression of NS5A. This in turn initiates STAT-3 and NF-κB pathway. The mechanism of ROS production initiates with the reduction of molecular oxygen, to form unstable superoxide O^{2-} reduces to a relatively stable hydrogen peroxide. Hydrogen peroxide is able to affect important cellular molecules to produce more free radicals (hydroxyl radicals) which enters the chain reaction [22–24]. Some major sources of ROS are;

Sources of ROS

Environmental factors	Mitochondrial factors
UV radiations	Electron transport chain
Viruses like RNA virus (HCV NS5A)	Oxidative deamination of organic amines
Allergens and industrial chemicals	(ER)-nucleus signal transduction pathway
Increased ozone, cigarette smoke & smog	–
Xenobiotic metabolism	–

On the other hand, cells have developed several protective mechanisms to prevent radical formation or to detoxify radicals. This series of reactions stop in two conditions. Either the substrates get depleted and milieu becomes anaerobic or when chain is broken up by the antioxidants. Antioxidants are found in foods or generated by the biological system itself. In most of the cases the harmful effects of ROS are eliminated by antioxidants. Antioxidants break the chain reaction of free radicals. Generally found antioxidants include vitamin E, vitamin C, and glutathione (GSH). In the case of viral liver diseases glutathione is a powerful antioxidant. These compounds have several mechanisms of action. For example, GSH can neutralize reactive oxygen radicals by transferring hydrogen to the reactive molecules, thus creating a more stable chemical structure [25,26]. But for the patients suffering from RNA virus diseases, like HCV, the levels of GSH and GR were found to be abnormal in plasma, epithelial fluid as well as in blood. The extent of depletion varies with the changes in severity, nature and duration of virus in the body [27–29].

It is not only the antioxidants like glutathione and glutathione-dependent enzymes whose normal concentration is altered by hepatitis C. The normal production and utilization of numerous essential micronutrients and trace metal is also disturbed by hepatitis C virus. These essential trace metals and micronutrients like zinc (Zn), copper (Cu), iron (Fe) and selenium (Se) play an important role in different liver physiological processes like enzymatic activities, redox status, inflammation, immune response and protein structure and function [30] . Selenium, in combination with alpha-lipoic acid and silymarin, has caused noticeable improvements in chronic HCV patients by upgrading the inflammatory response [31].

Essential micronutrients (Zn, Cu, Fe, and Se) might aggravate liver disease in case of deficiency, imbalance, or toxicity. Although the precise causes remain to be elucidated, there is evidence that cytokines might alter the levels of serum trace elements in viral hepatitis. It was reported that inflammatory cytokines are higher in HCV-infected individuals and increased Cu levels might result from inflammatory responses and they are directly related to the pathology developed in the liver by HCV [32].

The objective of the present study was to undertake in depth studies on the mechanism of action of different antioxidant enzymes and micronutrients (zinc, copper, iron, selenium) in the hepatitis C patients. The main target was to uncover the relationship between different micronutrients, antioxidant enzymes and hepatitis C virus by constantly monitoring the variations in the studied parameters at different viral load levels in genotype 3 HCV patients.

2. Patients and methods

2.1. Subject selection

200 random patients (82 F, 118 M), under treatment of different doctors from the start of treatment till the completion of therapy, were enrolled in this study. All the patients were diagnosed with hepatitis C. The patients were excluded from the study if they were positive for hepatitis B, other types of liver disease, renal diseases, diabetes mellitus or any other malignancies.

The study was retrospective and patient follow-up period ranged from the start of therapy up to 6 months to test the possibility of relapsing. None of the subjects have received any micronutrient supplementation for at least one year.

A hundred randomly selected individuals (50 F, 50 M) with the mean age of 24 years were without any clinical history or serological evidence of hepatitis, served as a healthy control group without any liver disease. The individuals with any possible association of oxidative stress due to the consumption of tobacco, alcohol or drugs were excluded from the study. Informed consent was taken from all the study patients and healthy controls. The study protocol was approved by the institution's human research committee.

2.2. Detection of HCV RNA

HCV RNA was isolated using the Roche AMPLICOR® Hepatitis C Virus (HCV) Test version 2.0. Detection of HCV RNA was done by using Light Cycler 2.0 real-time PCR (Roche Applied Sciences, Germany) (1).

2.3. Viral load

After detection of viral RNA, serum HCV RNA was quantitatively determined by AMPLICOR® HCV MONITOR™ (Roche Molecular Diagnostics) and expressed as a log 10 of copies of RNA per millimeter. A higher viral load indicates a higher level of infectiousness. The manufacturer instructions of the RNA kit were followed. This assay has a lower limit of 100 IU ml^{-1}. Quantitative PCR utilizes competitive RT-PCR involving two simultaneous reactions and incorporating an internal control. The target sequences chosen are generally in the conserved 5′ un-translated region of the HCV genome, which is important for sensitivity, specificity and reproducibility [33]. The internal control is a synthetic RNA molecule having the same primer recognition sequence as the HCV target sequence. Intensity of amplification of HCV RNA is then compared to the internal standard of known concentration to determine the relative concentration of HCV. The detection limit of current assays is as low as 100 copies/ml of viral RNA. The AMPLICOR Monitor assay is a modified RT-PCR assay undertaken in one tube. It is therefore simpler than conventional RT-PCR techniques and has a lower risk of contamination. The assay can detect 103 to 106 copies of HCV RNA per milliliter of patient serum. The accuracy of AMPLICOR Monitor assay may slightly vary in determining viral load for patients having different HCV genotypes. Within the reference range HCV RNA concentrations detected by the AMPLICOR assay correlated with PCR.

2.4. HCV genotyping

HCV genotyping was performed using a Cuto Flour, Third Wave Technology, USA. The third wave assay uses Cleavage Enzymes to recognize and cleave specific structures formed by the addition of two oligonucleotides (an Invader Oliga and Primary Probe) to the nucleic acid.

2.5. Micronutrient determination

2.5.1. Sample preparation and digestion

Before analysis, all the glasswares were washed in diluted nitric acid (10%) and rinsed. Standard solution (1000 μg l^{-1}) of selenium, copper, manganese, zinc and iron was obtained from Merck, Germany.

Digestion of the serum sample was done according to the already reported method by Safaralizadeh et al. [34,35]. According to which, serum (1 ml) was transferred to a Teflon beaker for mineralization, then 3 ml of $HNO_3/HClO_4$ (1:1 v/v) were added. The temperature of the sample was then brought gradually to boiling on a hot plate until fumes of $HClO_4$ appeared. Samples were then heated according to the following temperature/time scheme: 175 °C/60 min, 200 °C/60 min, and finally 250 °C/60 min. The mixture was then heated according to the following (temperature/time) scheme: 175 °C/60 min, 200 °C/60 min, and finally 250 °C for 60 min. The mixture was then left to cool down to room temperature. Then 10 ml of (6 N) HCl was added and the sample was heated again on the hot plate to 170 °C for 30 min.

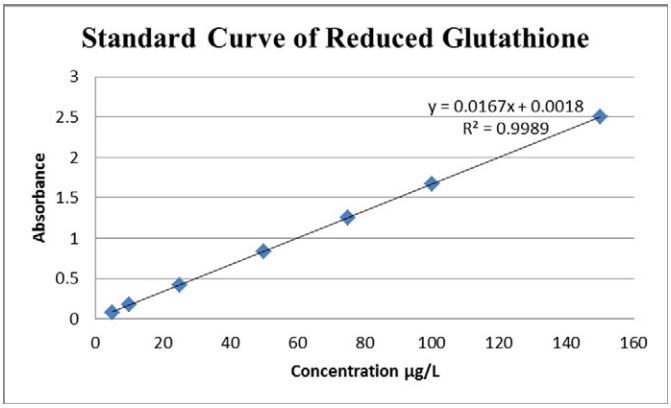
2.5.2. Sample analysis

The digested serum samples were analyzed for the micronutrients by using an atomic absorption spectrophotometer, model Analytic Jena (Vario III).

2.5.3. Estimation of glutathione and its enzyme activity

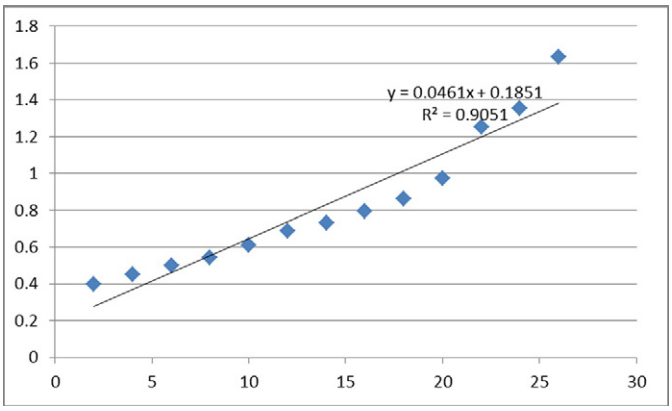
Reduced glutathione was determined by the method of Moron et al. (1979) and glutathione peroxidase activity was measured by methods of Aydin et al. [36–38]. GSH was measured by the method of Tietze [39]. GSH reacts with Ellman's reagent (5,5-dithio bis (nitrobenzoic acid) or DTNB) to produce a chromophore TNB with a maximal absorbance at 412 nm and oxidized glutathione GSSG. The amount of glutathione measured represents the sum of reduced and oxidized glutathione in the sample ([GSH]t = [GSH] + 2 × [GSSG]). The rate of absorbance change (ΔA 412 nm/min) is made to be linear for the convenience and consistence of measurement, and is linearly proportional to the total concentration of GSH. The concentration of an unknown (sample) is determined by calculating from the linear equation generated from several standards of glutathione.

Glutathione peroxidase activity was measured by methods of Aydin et al. Prepare reaction mixture with 50 mmol/L TRIS buffer (pH 7.6) and add to it 1 mmol/L Na2EDTA + 2 mmol/L reduced glutathione + 0.2 mmol/L NADPH + 4 mmol/L sodium azide + 1000 U glutathione reductase (1 U = 11 μg/ml). The chemicals involved reaction mixture and 8.8 mmol/L of hydrogen peroxide. 20 μl of serum was added in a test tube and then 980 μl of reaction mixture was added and then incubated for 5 min at room temperature (37 °C). Reaction was started by addition of 0.5 ml of 8.8 mmol/l hydrogen peroxide. Then decreased absorbance was taken at 340 nm for 3 min, every 1 min.



Y = 0.0167 X − 0.0098
X = Concentration of GR.
Y = absorbance of sample.

Glutathione was assayed according to the method of Tietze [39]. 0.1 ml tissue homogenate of each group was taken in test tubes. 2.4 ml of 0.02 M EDTA was added in each test tube and kept in ice bath for 10 min. Then 2.0 ml of dH_2O (distilled water) was added in each test tube. 0.5 ml 50% TCA was added and kept in ice bath for 10–15 min. The mixture was centrifuged at 3000–3500 rpm for 10 min. 1.0 ml supernatant was taken is separate tubes and added 2.0 ml 0.15 M Tris HCl and 0.05 ml DTNB. The mixture was vortexed and absorbance was taken after 2–3 min at 412 nm. The absorbance was compared with standard curve generated by the known GSH level. The GSH will be measured in μg/ml.



Standard curve of GSH (μg/ml)

3. Results

In the present study HCV patients are divided into three major groups according to their viral load levels. In the mild group, the range of viral load was 200,000–1,000,000. The moderate group included the patients with the viral load ranging from 1,000,000 to 5,000,000 and the severe group has a range of 5,000,000–25,000,000.

The concentration of Cu, Se and Fe (Table: 1.1) was found to be significantly increased, with the increase in HCV RNA levels, as compared to the control ($P < 0.05$). In the mild, moderate and severe HCV RNA groups the mean of Cu was 96.4 mg/L, 102.2 mg/L and 106.3 mg/L respectively. In the mild, moderate and severe HCV RNA groups the mean of Fe was 103.3 μg/ml, 103.8 μg/ml and 101.9 μg/ml respectively. In the mild, moderate and severe HCV RNA groups the mean of Se was 15.89 μg/ml, 12.87 μg/ml and 14.25 μg/ml, respectively.

On the other hand, the concentration of Mn (Table 1.1) was found to be significantly decreased in the mild group and it elevated in the moderate and severe groups as compared to the control ($P < 0.05$). In the mild, moderate and severe HCV RNA groups the mean of Mn was 7.26 mg/ml, 8.50 mg/ml and 9.61 mg/ml, respectively. The concentration of Zn was found to be decreased significantly, as compared to the control ($P < 0.05$). In the mild, moderate and severe HCV RNA groups the mean of Zn was 65.7 mg/L, 76.02 mg/L and 73.9 mg/L, respectively.

The serum concentrations of glutathione reductase (Table 1.2) were found to be significantly increasing with the increase in the levels of HCV RNA ($P < 0.05$). In the mild, moderate and severe HCV RNA groups the mean of GR was 6.377 μg/L, 6.44 μg/L and 6.406 μg/L, respectively. However, the serum concentration of GSH and GPx (Table 1.2) were found to be significantly depleting, with the increase in HCV RNA levels, and as compared to the control ($P < 0.05$).

The study identified the mutual correlation between the selected parameters by applying in two-tailed Pearson correlation matrix analysis, Table 1.3. Copper is correlated with selenium (0.492**), iron (.365**), GSH (−.418**) GR (.437**), and GPx (−.489**). Manganese showed a significant correlation with GR (.552**), GPx (−640**), copper (.492**), iron (.518**) and GSH (.448**). Iron was significantly correlated with GR (.552**), GPx (−640**), GSH (−.564**), Copper (.492**), Selenium (.518**), Manganese (−.385**). Selenium showed a positive correlation with copper (.492**), iron (.518**) and GR (.435**) and inverse relationship with GSH (−.429**) and GPx (−.463**). Zinc was significantly correlated with GR (−.330**), GPx (.439**) and GSH (.492**).

4. Discussion

Antioxidants like the glutathione family including glutathione, glutathione peroxidase and glutathione reductase act as biological indicators for the assessment of many liver abnormalities like hepatitis C and HCC [39,40]. In a study it was found that in HCV patients excessive mitochondrial ROS are produced and as a result of obvious undesired changes in the redox status of the patients and the levels of GSH, GPX are found [41].

Reduced levels of selenium in this study can also be explained by the fact that selenium is used as a co-factor for glutathione peroxidase enzyme [27]. In HCV patients reduced levels of Se-dependent GPX and reduced antioxidative ability are linked with hepatitis C and the reduced micronutrient levels [42].

The essential micronutrients (Zn, Cu, Fe, and Se) might aggravate liver disease in case of deficiency, imbalance, or toxicity. Although the precise causes remain to be elucidated, there is evidence that cytokines might alter the levels of serum trace elements in viral hepatitis. It was reported that inflammatory cytokines are higher in HCV-infected individuals and increased Cu levels might result from inflammatory responses and they are directly related to the pathology developed in the liver [43]. In the present study, Table 1.1, the serum levels of essential micronutrients like copper, selenium and iron were found to be significantly elevated and manganese and zinc concentrations were reduced in the present study. In patients with HCV there is an inverse relation between total serum zinc concentration and serum copper, and direct association with plasma levels of retinol-binding protein

Table 1.1
Serum concentrations of micronutrients (Zn, Fe, Mn, Se and Cu).

Parameter	Healthy control	Viral load of HCV patients			Sig.
		Mild (20,000–100,000 IU/ml)	Moderate (100,000–500,000 IU/ml)	Severe (above 500,000 IU/ml)	
Zn (mg/l)	88.9 ± 3.33	65.7 ± 10.9	76.02 ± 18.05	73.9 ± 25.12	0.00
Fe (μg/ml)	71.4 ± 22.8	103.3 ± 12.3	103.8 ± 6.10	101.9 ± 10.4	0.00
Mn (mg/ml)	11.24 ± 0.925	7.26 ± 1.80	8.50 ± 2.49	9.61 ± 1.08	0.001
Se (μg/ml)	6.41 ± 1.7	15.89 ± 5.17	12.87 ± 4.11	14.25 ± 3.47	0.00
Cu (mg/L)	65.5 ± 20.01	96.4 ± 21.8	102.2 ± 17.1	106.3 ± 5.93	0.00

Table 1.2
Serum concentrations of glutathione and glutathione-dependent enzymes.

Parameter	Healthy control	Viral load of HCV patients			Sig.
		Mild (20,000–100,000 IU/ml)	Moderate (100,000–500,000 IU/ml)	Severe (above 500,000 IU/ml)	
GR (μg/l)	5.88 ± 0.325	6.37 ± 0.126	6.41 ± 0.115	6.44 ± 0.208	0.00
GPx (μmol/ml)	8.15 ± 1.23	4. 72 ± 0.175	4.68 ± 4.27	4.57 ± 0.06	0.00
GSH (μg/ml)	10.3 ± 0.97	3.01 ± 0.824	1.81 ± 0.63	0.71 ± 0.67	0.00

Table 1.3
Pearson correlation matrix (2-tailed) between the studied parameters of HCV patients.

	GR	GPx	Cu	Zn	Se	Fe	Mn	GSH
GR	1	−.546**	.437**	−.330*	.435**	.552**	−.403**	−.680**
GPx		1	−.489**	.439**	−.463**	−.640**	.471**	.809**
Cu			1	−.076	.492**	.365**	−.175	−.418**
Zn				1	−.244	−.176	.267*	.412**
Se					1	.518**	−.108	−.429**
Fe						1	−.385**	−.564**
Mn							1	.448**
GSH								1

** Correlation is significant at the 0.01 level (2-tailed).

(RBP) and total protein. The changes in serum and urinary zinc may be related to changes in the manner by which zinc is bound to serum proteins, particularly albumin. Total serum copper concentrations are found to be increased and this increase was accounted for entirely by increases in the concentration of ceruloplasmin copper [44] (Fig. 1.2). The present study, Table 1.3, also confirmed this association between zinc and copper and their altered concentrations in HCV patients as compared to the healthy subjects. The irregular levels of these micronutrients, significantly reduced concentrations of plasma zinc (Zn); higher copper (Cu), and iron (Fe), may increase oxidative stress [38] and hence, they can be used as indicators of oxidative stress in the HCV patients. (See Fig. 1.1.)

Excessive concentration of iron (Table 1.1) in the hepatitis C patients is associated with the HCV and HCV induced ROS. Due to this RNA virus, the levels of 8-hydroxydeoxy-guanosine, 8-OHdG, significantly increases and the levels of hepcidin lowers. The elevated levels of 8-OHdG and reduced hepcidin are responsible for the iron overload in the hepatitis patients [45]. Altered iron metabolism in the hepatitis C

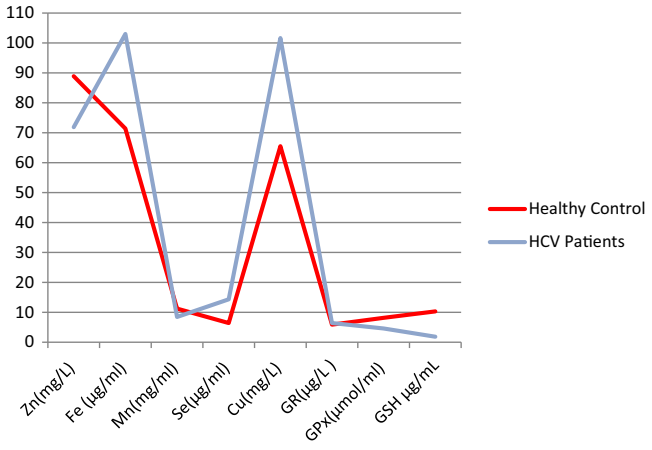


Fig. 1.2. Comparison between HCV positive patients and controls.

patients can be associated with the decline in catalase activity as it is the cofactor of catalase (Fig. 1.2).

Due to hepatitis C virus, metabolism of zinc is disturbed (Table 1.1). HCV resulted in impaired synthesis of albumin and decreased level of serum zinc concentration [46]. Similarly, abnormal protein metabolism, carbohydrate metabolic disturbances, abnormal serum levels of some liver enzymes (Table 1.4) like glutamine alkaline transferase, glutamine pyruvate transferase, alanine aminotransferase, and a decrease in hepatic antitoxic role due to HCV are also responsible for abnormal zinc metabolism [47].

In hepatitis C, due to oxidative stress, the levels of antioxidant (vitamins), antioxidant enzyme GPx and ALT, AST, manganese, and zinc decreased and GR, copper, selenium and iron increased. From the

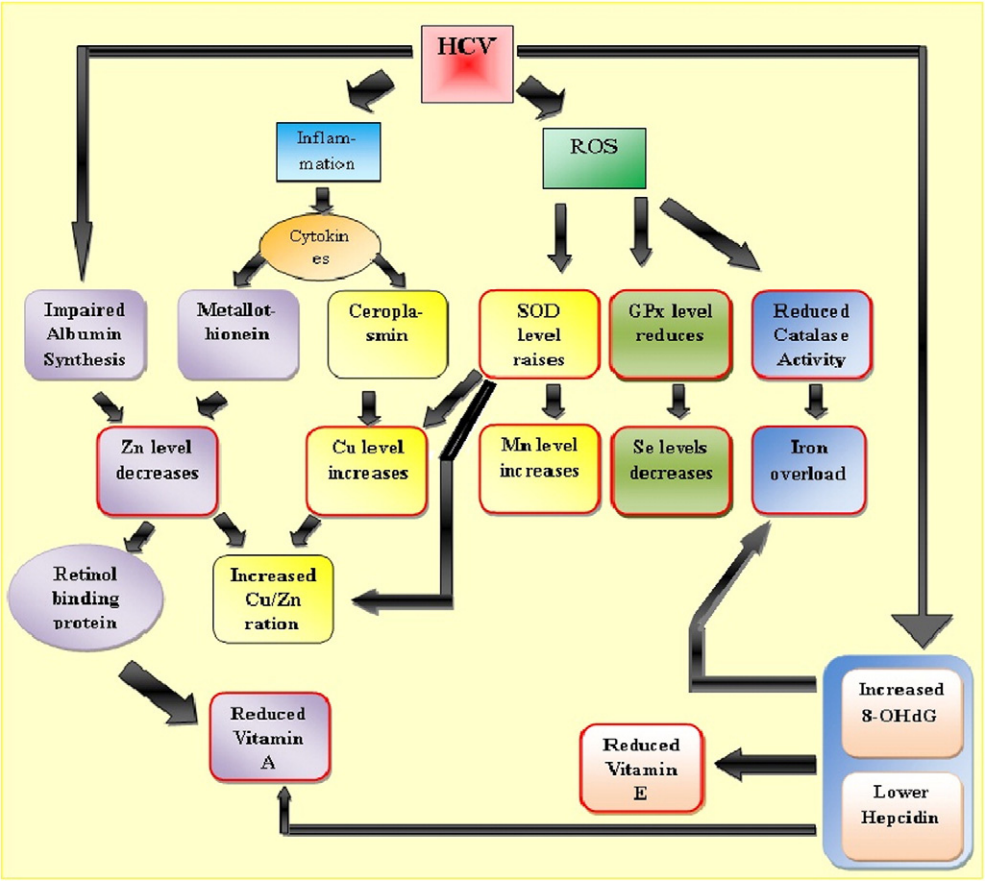


Fig. 1.1. Mechanism of correlation between antioxidants and micronutrients leading to the reduced working abilities of antioxidant defense system due to HCV.

Table 1.4
HCV patients and healthy control characteristics at the baseline.

Characteristics	Group A (patients) n = 200	Group B (healthy individuals) n = 100
Male	118 (59%)	50 (50%)
Mean age, years ± SD	36.1 ± 10.8	31 ± 9.8
Female	82 (41%)	50 (50%)
Mean age, years ± SD	37.1 ± 9.21	24 ± 9.76
ALT	78.4 ± 22.5	23.4 ± 5.69
AST	82.4 ± 20.44	26 ± 5.66
TB	0.7 ± 0.90	0.4 ± 0.185
HCV RNA level IU/ml	4.594E6 ± 9.28E6	–
HCV genotype	3	–

Pearson correlation matrix (Table 1.3) the relationship between different micronutrients and antioxidants showed that in a biological system there is a proper sequence of execution of events and these events are interdependent on each other.

5. Conclusion

Oxidative stress is a common pathogenetic mechanism that is responsible for the initiation and progression of hepatic damage after exposure to hepatitis C virus. We aimed to investigate the relationship between viral load and micronutrients and glutathione and glutathione-dependent enzymes as oxidative stress markers among HCV genotype 3 patients and none of the subjects have received any micronutrient supplementation for at least one year. Consequently, we achieved a high significant correlation between the study parameters and found it a reliable indicator of hepatic cellular injury in the hepatitis C patients. So far, these parameters are hardly focused in clinical studies to control oxidative stress and the patients are mostly treated to clear the hepatitis C virus quantitatively as well as qualitatively. As there was a strong association of the selected parameters with the onset and progression of disease, they can be used as a successful therapeutic biomarker to diagnose the status of infection. Thus, the use of these markers will be the least expensive and significantly reliable tool to check the presence of hepatitis C and stage of the disease.

In conclusion, this study showed a significant and positive association between serum GSH, GPx, GR, copper, manganese, zinc, selenium, iron levels and different levels of viral load in HCV patients of genotype 3. This association, in fact, has a strong impact on a modified immune response, steatosis and fibrosis, mitochondrial damage, altered protein structure and function, activation of inflammatory cells, apoptosis and necrosis in HCV patients.

Acknowledgments

Collective and individual acknowledgement is also given to my professors and colleagues at the Institute of Molecular Biology and Biotechnology, The University of Lahore.

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