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Evaluation of Carbonylated Proteins in Hepatitis C Virus Patients

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Abstract

Carbonylated proteins are irreversible posttranslational oxidative modifications, which may interfere with the normal homeostasis of cell growth inducing liver cirrhosis and risk of malignancy.

Objective: To determine plasma levels of carbonylated proteins and total antioxidant capacity and evaluate their role in HCV hepatitis patients and HCV induced liver cirrhosis before and after antiviral therapy; interferon and ribavirin.

Methods: This study included twenty chronic hepatitis C patients with cirrhotic changes, twenty chronic hepatitis C patients without cirrhotic changes and before taking interferon therapy and Fifteen chronic hepatitis C patients without cirrhotic changes and after taking antiviral therapy; interferon (PEG-IFN α2a 180-µg/week and Ribavirin 800 mg capsule one time daily for 24 weeks). Twenty male healthy individuals were included as the control group (age, and body mass index matched). All patients were taking liver support supplements containing vitamins; C, E, folic acid and carotenoids.

Results: There was a highly significant increase (p value 0.00001) in plasma carbonylated protein level in cirrhotic patients (44.9 ± 5.63 nM/dL) as compared to the control group (22.3 ± 3.35 mM/L). TAC in serum in cirrhotic patients was significantly decreased to 0.765 ± 0.249 mM/L as compared to all other groups. These patients were taking antioxidants vitamins (vitamin C, carotenoids and vitamin E), and other supplements known to have antioxidant effects (silimaryn, trace elements), which did not increase their TAC.

Conclusions: Carbonylated proteins may play a role in HCV induced liver cirrhosis. The currently used antioxidants did not increase the antioxidant capacity of plasma. New antioxidants as well as inducers of antioxidant enzymes may be helpful in increasing TAC and prevention of formation of carbonylated proteins and liver cirrhosis.

Keywords: Carbonylated proteins; Total antioxidant capacity; HCV hepatitis; Liver cirrhosis; Interferon; Ribavirin

Introduction

Egypt has a high prevalence of Hepatitis C Virus (HCV), and its complications as liver cirrhosis and hepatocellular carcinoma. Hepatitis C virus infection represents a major health issue worldwide due to its burden of chronic liver disease and extra hepatic manifestations. HCV hepatitis is treated with antiviral therapy, which includes interferon and ribavirin. The therapy usually extends for 24 weeks or more. Yet, in Egypt, liver cirrhosis is still a complication of HCV hepatitis, even in patients who received the antiviral therapy, which may suggest that additional factors contribute for the cirrhosis. HCV variants with reduced susceptibility to interferon can occur naturally, even before treatment begins [1].

HCV causes oxidative stress by a variety of processes, such as activation of prooxidant enzymes, weakening of antioxidant defenses, organelle damage, and metals unbalance. A focal point, in HCV-related oxidative stress onset, is the mitochondrial failure. Mitochondria have a central role in energy production, metabolism, and metals homeostasis, mainly copper and iron. Furthermore, mitochondria are direct viral targets, because many HCV proteins associate with them. They are the main intracellular free radicals producers and targets [2].

Oxidative stress is a condition of oxidant /antioxidant disequilibrium where there is overproduction of Reactive Oxygen Species (ROS) on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other side. Examples of non-enzymatic antioxidants are; bilirubin, vitamin C, vitamin E, β- carotene, and flavonoids. The resulting oxidative stress damages various cellular component including lipids, proteins and nucleic acids inhibiting their normal function. ROS damage to cellular component plays an important role in numerous diseases including cancer, metabolic syndrome, atherosclerosis, cardiovascular diseases, neurological disorders, Alzheimer disease, diabetes mellitus, aging, autoimmune diseases, and chronic inflammatory diseases [3].

Oxidative stress induces reactions in cellular and blood lipids and proteins. ROS can mediate intra- and intermolecular cross-linking of peptides and proteins and fragmentation of polypeptide chains. In addition, protein carbonyls are formed because of the oxidative modifications of proteins. Carbonyl groups are introduced into proteins by two distinct mechanisms: oxidative (direct) and non-oxidative (indirect). Oxidative mechanisms, which are metal catalyzed, involve the direct reaction of certain reactive oxygen species (e.g., hydrogen peroxide and lipid hydro peroxides) with protein side chains. Non-oxidative carbonylation of proteins involves the reaction of the nucleophilic centers in cysteine, histidine or lysine residues with reactive carbonyls (RCOs). RCOs are carbonyl-containing malondialdehyde, acrolein and carbohydrates (e.g., glyoxal, methylglyoxal) [4].

Damage of proteins by oxidative stress could be involved in inflammation-related carcinogenesis. Detection of elevated levels of protein carbonyls in blood or tissues indicates generally a disease associated dysfunction and defective immunological responses and macrophages functions [5].

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The aim of this work was to determine the plasma level of carbonylated proteins and total antioxidant capacity and evaluate their role in HCV hepatitis patients and HCV induced liver cirrhosis. In addition, the effect of antiviral therapy; interferon and ribavirin on their levels in plasma was evaluated.

Subjects and Methods

Patients and controls

This study included 55 male patients with chronic hepatitis C from the internal medicine department, Damietta, Egypt. The patients were already diagnosed by viral markers.

Twenty male healthy individuals were included as the control group (age, and body mass index matched; Table 1).

Patients were divided into 3 groups:

Group 1: Twenty chronic hepatitis C patients with cirrhotic changes diagnosed by Sonography.

Group 2: Twenty chronic hepatitis C patients without cirrhotic changes and before taking interferon therapy**.**

Group 3: Fifteen chronic hepatitis C patients without cirrhotic changes and after taking antiviral therapy; interferon (PEG-IFN α2a 180-µg/ week and Ribavirin 800 mg capsule one time daily for 24 weeks).

All patients were taking liver support supplements containing vitamins; C, E, folic acid and carotenoids.

Exclusion criteria

Subjects suffering from any systemic disease or autoimmune disease were excluded from the study. In addition, subjects with positive HBV or have taken HBV vaccine, smokers and obese subjects with BMI >30 kg/M^2 were excluded from the study.

Only patients with HCV hepatitis were included in this study.

Written medical consent was signed by all participants, and the protocol of the work was approved by the Ethical Committee of Al-Azhar University (Table 1).

All the patients were taking orally liver supporting drugs: Silymarin; 400 mg, vitamin C 250 mg, vitamin E; 20 mg, selenium36.6µg, zinc7.3 mg and Glutathione 250 mg, daily.

		Weight (kg)	Height (cm)	BMI (kg/M^2)
Control	Mean ± SD	88.7 ± 7.5	177.85 ± 6.7	28.02 ± 1.8
Group 1	Mean ± SD	84.2 ± 10.7	176.85 ± 7.9	26.81 ± 2.1
Group 2	Mean ± SD	83.8 ± 9.7	178.5 ± 9.8	26.29 ± 2.5
Group 3	Mean ± SD	85.4 ±7.3	182 ± 9.7	25.8 ± 2.4

Table 1: Weight, height and BMI of patients and control group.

	Control	Group 1	Group 2	Group 3
Mean ± SD	3.9 ± 0.38	2.04 ± 0.567	4.04 ± 0.377	3.8 ± 0.376
Control	t-test P-value	12.417 0.0003*	-0.835 0.409	1.133 0.265
HCV before interferon	t-test P-value	-13.1 0.0002*	----------	----------
HCV after interferon	t-test P-value	----------	1.914 0.065	----------

*Highly significant

Table 2: Serum albumin in different groups (g/dL).

Blood samples

Blood samples were obtained from patients and healthy controls after 12 hours of fasting.

Two mL in polypropylene tubes, left to clot for 20 minutes at 37°C, centrifuged at 3000 g for 10 minutes and serum was separated and used for determination of ALT, AST, alkaline phosphatase, and albumin.

Four mL in polypropylene tubes containing sodium citrate as an anticoagulant for determination of carbonylated protein level and TAC in plasma. Blood was centrifuged for 10 minutes at 3000 g immediately after collection and plasma was removed and stored at <-80°C till assay.

Methods

Serum albumin, Alanine Transaminase (ALT), Aspartate Transaminase (AST) and Alkaline Phosphatase (ALP) were assayed on Roche/Hitachi 902 chemistry auto analyzer, by using kits supplied by Roche Diagnostic, Germany [6].

Total plasma antioxidative capacity was determined by the reaction of antioxidants in the sample with a defined amount of exogenously provided hydrogen peroxide (H_2O_2). The antioxidants in the sample eliminate a certain amount of H_2O_2, The residual H_2O_2 was determined calorimetrically by an enzymatic reaction which involves the conversion of 3, 5, dichloro-2-hydroxy benzene sulphonate to a colored product which was measured at 505 nm [7].

Carbonylated proteins in plasma were measured using Cayman's Protein Carbonyl Colorimetric Assay Kit. The kit utilized the DNPH reaction as described by Levine et al. [8]. After incubation of DNPH with samples, trichloroacetic acid 10% (w/v) was added and samples were centrifuged at 11,000 g in a cooling centrifuge. The precipitates were washed with a mixture of ethanol/ethyl acetate, 1:1 (v/v) three times and then dissolved in guanidine hydrochloride 6M (pH 2.3). The absorbance was then measured at 360 nm and the concentration of CO groups was calculated using the molar extinction coefficient (e) of 22,000 cm^{-1} M^{-1}.

Protein concentration of the samples was determined by the Bradford's assay [9] using a microplate reader (Bio-Rad 3550; Bio-Rad, Hercules, CA) at 595 nm and BSA as standard.

Statistical analysis

Results were expressed as Mean ± Standard Deviation (SD). Comparison between groups was done using Student's t test with significance defined as $p \leq 0.05$.

Results

Serum albumin

Serum albumin level was 3.9 ± 0.38 g/dl in the control group. Serum albumin levels were 2.04 ± 0.567, 4.04 ± 0.377 and 3.8 ± 0.376 g/dl in-group 1, 2 and 3 respectively (Table 2). There was a highly significant decrease in albumin level in cirrhotic patients (group 1) compared to all other groups.

Plasma total antioxidant capacity

Total plasma antioxidant capacity levels were 1.55 ± 0.272, 2.04 ± 0.290 and 2.63 ± 0.193 mmol/liter in the control group, group 2 and group 3 respectively. Plasma TAC in chronic HCV hepatitis patients before and after antiviral therapies were therefore, significantly increased. Plasma TAC in group 1 (cirrhotic patients) was significantly decreased to 0.765 ± 0.249 mmol/liter as compared to all other groups (Table 3).

	Control	Group 1	Group 2	Group 3
Mean ± SD	1.55 ± 0.272	0.765 ± 0.249	2.04 ± 0.290	2.63 ± 0.193
Control	t-test P-value	9.545 0.0004*	-5.453 0.002*	-13.067 0.0003*
HCV before interferon	t-test P-value	-14.88 0.0002*	----------	----------
HCV after interferon	t-test P-value	----------	-6.861 0.002*	----------

*Highly significant

Table 3: Total anti-oxidant capacity in plasma (mmol/litre).

	Control	Group 1	Group 2	Group 3
Mean ± SD	22.3 ± 3.35	44.9 ± 5.63	27.22 ± 2.92	24.9 ± 2.83
Control	t-test P-value	-15.414 0.00001*	-4.916 0.003*	-2.43 0.21
HCV before interferon	t-test P-value	12.475 0.0003*	----------	----------
HCV after interferon	t-test P-value	----------	2.312 0.027	----------

*Highly significant

Table 4: Plasma carbonylated proteins in different groups (nM/dL).

		AST U/dl	ALT U/dl	AST/ALT (AAR)	ALP U/L
Control	Mean ± SD	25.8 ± 4.3	25.1 ± 3.7	1.03 ± 0.1	79.7 ± 22.3
Group 1	Mean ± SD	24.8 ± 3.7	25.05 ± 3.1	0.99 ± 0.06	89.45 ± 27.8
Group 2	Mean ± SD	124.5 ± 27.4*	131.35 ± 20.8*	0.97 ± 0.04	218.45 ± 50.3*
Group 3	Mean ± SD	25.8 ± 3.7	27.87 ± 3.8	0.93 ± 0.08	88 ± 22.2

*Statistical significant when compared to the control group (p-value ≤ 0.01)

Table 5: Serum aminotransferases, alkaline phosphatase (ALP) and AST/ALT ratio (AAR) in control group and patients.

Plasma carbonylated proteins

Carbonylated proteins level in plasma of HCV patients with cirrhosis was 44.9 ± 5.63 nM/dL. Carbonylated proteins levels in control group, and group 3 were 22.3 ± 3.35, and 24.9 ± 2.83 mM/L respectively. There was a highly significant increase in carbonylated protein level in cirrhotic patients compared to all other groups. Carbonylated proteins levels in-group 2 was 27.22 ± 2.92 nM/dL, showing a significant increase as compared to control group and group 3 (Table 4).

Serum aminotransferases, alkaline phosphatase and AST/ALT ratio (AAR) in control group and patients

Serum levels of AST, ALT and ALP in HCV hepatitis patients were significantly high (AST; 124.5 IU/dl, ALT; 131.35 IU/dl, ALP; 218.45 ± 50.3 U/L) when compared to the control group (AST; 25.8 ± 4.3 IU/dl, ALT; 25.1 ± 3.7 IU/dl, 79.7 ± 22.3 U/L). The levels of serum aminotransferases and ALP in patients taking interferon and ribavirin therapy, decreased to near control values. Interferon therapy in these patients decreased the inflammatory process in hepatocytes. AAR ratio in cirrhotic patients in this study was 0.99 (24.8/25.05). The ratio was 1.03 ± 0.1, 0.97 ± 0.04 and 0.93 ± 0.08 in control group, group 2 and group 3 respectively. There was no statistical significance between the values of all groups (Table 5).

Discussion

Serum albumin

HCV hepatitis is an acute to chronic inflammation of hepatocytes, which may develop into liver cirrhosis. The levels of serum albumin were significantly decreased in patients with HCV hepatic cirrhosis (2.04 g/dl) compared to the control group (3.94 g/dl). The changes in the level of albumin were not significant in HCV hepatitis patients without cirrhosis. The low level of serum albumin and cirrhotic changes in liver parenchyma may lead to ascites and edema in lower limbs. In humans, albumin synthesis takes place only in the liver. Albumin is not stored by the liver but is secreted into the portal circulation as soon as it is manufactured. Under physiological conditions, albumin may have significant antioxidant potential. This may be related to the abundance of sulfhydryl (-SH) groups on the albumin molecule [10].

Albumin therapy is recommended in refractory ascites not responsive to diuretics. Indications for albumin therapy are linked to the antioxidant activity of albumin. Albumin exchange has emerged as promising liver support therapies for liver failure and other toxic syndromes. They are designed to remove a broad range of blood-borne toxins and to restore normal functions of the circulating albumin by replacing defective forms of albumin and albumin molecules saturated with toxins with normal albumin [11].

Serum aminotransferases and alkaline phosphatase

The most commonly used markers of hepatocyte injury are AST (cytosolic and mitochondrial forms), and ALT (cytosolic). As markers of hepatocellular injury, AST and ALT also lack some specificity because they are found in other tissue. HCV is one of the risk factors for hepatocellular carcinoma. AST/ALT ratio (AAR) may be used as a possible surrogate marker for identifying patients at high risk for developing hepatocellular carcinoma. AAR > 1.4 might be a useful tool to identify candidates at high risk for HCC. This ratio in cirrhotic patients in this study was 0.99 (24.8/25.05). The ratio was 1.03 ± 0.1, 0.99 ± 0.06, 0.97 ± 0.04 and 0.93 ± 0.08 in control group, group 1, group 2 and group 3 respectively. There was no statistical significance between the values of all groups. AAR in all patients was lower than the cut value of suspecting malignancy [12].

Alkaline phosphatase in serum of HCV hepatitis patients without antiviral therapy, and using only hepatic supportive drugs was significantly increased. inflammation of the bile ducts occurs frequently in chronic hepatitis C, and decreases in response to IFN therapy [13].

Carbonylated proteins

Protein carbonyl group can be generated directly by amino acid oxidation or indirectly by forming adduct with lipid peroxidation product. Carbonylation is an irreversible post-translation modification that often leads to loss of protein function. Carbonylated proteins are a stable marker of severe oxidative stress because damage to the protein structure is irreversible and may cause an inhibition of their enzymatic activity or an increased susceptibility to proteolysis [14].

Detection of elevated levels of protein carbonyls in blood or tissues, indicates generally a disease associated dysfunction and defective immunological responses and macrophages functions [15].

The carbonylated modification of protein may lead to proteins that are recognized as non-self by the immune system. The resultant antibody will cross react with normal tissue proteins so initiating autoimmune diseases [16]. Chemicals, which increase ROS production and oxidative stress damage to protein, may lead to dys-morphogenesis and teratogenesis in fetus [17].

ROS interfere with the expression of number of genes, signal transduction pathway, and are thus instrumental in the process of carcinogenesis. The abnormal behaviors of neoplastic cells can be traced to an alteration in cell signaling mechanisms such as cytoplasmic receptor for tyrosine kinas, altered level of growth factor, proteins of transcription apparatus, protein involved in cell cycle. All these protein can be oxidized and carbonylated by ROS. Permanent modification

of genetic materials and protein responsible for DNA replication and repair and control of cell cycle by oxidative damage represents the first step in carcinogenesis. The significantly high levels of carbonylated proteins in HCV cirrhotic patients may suggest that they are at risk of hepatocellular carcinoma. These protein oxidations should be treated before they are initiated at the stage of HCV hepatitis [18].

Total anti-oxidant capacity

Assay of Total anti-oxidant capacity measures a complex of non-enzymatic antioxidants present in blood, which include exogenous antioxidants such as ascorbic acid, α tocopherol, β carotene and polyphenols. It also measures the endogenous antioxidants such as reduced glutathione, uric acid, and bilirubin. All patients were taking antioxidants including Silymarin and ascorbic acid, as routine since they were diagnosed as hepatitis patients even before the cause of hepatitis was determined for about three months before enrolled in this study. Silymarin flavonolignans used by patients in this study as polyphenol antioxidant are rapidly absorbed [19]. Silymarin displays anti-inflammatory effects on T lymphocytes in vitro and modest nonspecific immunomodulatory effects in vivo [20]. Ascorbic acid inhibits intracellular ROS generation and reduces the ethanol-induced inflammation in hepatocytes [21].

Taking antioxidants may explain the high level of blood total antioxidants in HCV hepatitis patients as compared to the control group. Supplementation of exogenous antioxidants did not increase antioxidant status in cirrhotic patients. The low levels of TAC in patients with liver cirrhosis may be either due to decrease in the level of plasma albumin or in the synthesis of glutathione and other endogenous antioxidants. In addition, it may be due to increased ROS production in the body.

New antioxidants as well as inducers of antioxidant enzymes; Mn/superoxide dismutase and catalase, may be helpful in increasing TAC and prevention of formation of carbonylated proteins. Drugs, which can increase specifically proteolysis of carbonylated proteins, may be tried in liver cirrhosis patients [22].

Conclusion

Carbonylated proteins are irreversible posttranslational modification, which may interfere with the normal homeostasis of cell growth inducing liver cirrhosis and risk of malignancy [23-25]. There was a highly significant increase (p value 0.00001) in carbonylated protein level in cirrhotic patients (44.9 ± 5.63 n M/dL) as compared to the control group (22.3 ± 3.35 mM/L) [26-28].

TAC in plasma in cirrhotic patients was significantly decreased to 0.765±0.249 mM/L as compared to all other groups [29]. These patients were taking antioxidants vitamins (vitamin C, carotenoids and vitamin E), and other supplements known to have antioxidant effects (silimaryn, trace elements), which did not increase their TAC [30,31].

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