Role of Oxidative Stress and Heme Oxygenase-1 Expression in Liver Steatosis with and without Chronic Hepatitis C Virus Infection

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Abstract:Background and aims: Heme oxygenase-1 (HO-1), the rate limiting enzyme in heme catabolism and its three by-products have recently received overwhelming research attention because of their anti-inflammatory and cytoprotective effects. Gene expression of HO-1 is upregulated in response to various oxidative stress stimuli. Patients with non alcoholic fatty liver disease (NAFLD) and chronic hepatitis C virus infection (CHC) appear to modify the production of oxidants which may affect the disease progression. We aimed to assess the level of HO-1 gene expression in the liver tissues in NAFLD patients with and without HCV in an attempt to shed light on its antioxidant potential and whether it has any relation to the disease severity and progression. **Patients and methods:** The study included 65 patients, 36 of them had CHC and 29 had NAFLD, as well as 20 healthy volunteers as controls. Physical, clinical examination, abdominal ultrasound, anthropometric measurements and liver biopsy were done for all participants. The level of HO-1 gene expression in the liver tissue and peripheral blood was assayed by reverse-transcriptase polymerase chain reaction (RT-PCR.). The association between the hepatic HO-1 mRNA expression and its expression in the peripheral blood, GSH, LIC, HOMA index, and the disease severity by means of NAFLD grade and fibrosis stage were assessed. **Results**: HO-1 mRNA expression in the liver biopsies of NAFLD patients was significantly higher than that of the controls. Patients with CHC showed significantly lower HO-1 mRNA expression than thecontrols. On comparing the level of expression in both patient groups, significantly higher levels were observed in NAFLD patients. Significant associations have been observed between hepatic HO-1 mRNA expression and its expression in the peripheral blood, GSH, HOMA-IR, LIC, NAFLD grade and fibrosis stage. **Conclusion:**Steatohepatitis is associated with oxidative stress; the body defence againstit could be the determinant for disease progression. Bearing in mind its protective antioxidant and possible pro-oxidant effect, proper modulation of the HO-1 pathway may provide a new therapeutic approach to arrest further HCV disease evolution.

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1.Introduction:

Non alcoholic fatty liver disease (NAFLD) encompasses a wide spectrum of liver disorders characterized by histologic lesions, ranging from simple steatosis to cirrhosis. The so-called non-alcoholic steatohepatitis (NASH) is a subtype of NAFLD in which steatosis is accompanied by hepatocytes ballooning and necrosis with or without Mallory hyaline bodies and fibrosis***(Puri and Sanyal, 2012)***.

It is not yet understood why simple steatosis develops in some patients, whereassteatohepatitis and progressive disease develop in others; differences in body-fat distributionor antioxidant systems, possibly in the context of a geneticpredisposition, may be among the explanations***(Day, 2006; Petta et al., 2009)***. Theprimary metabolic abnormalities leading to lipid accumulationare not well understood, but they couldconsist of alterations in the pathways of uptake, synthesis,degradation, or secretion in hepatic lipid metabolismresulting from insulin resistance ***(Musso et al., 2010).***

Coexistence of hepatic steatosis and HCV infection is associated with an accelerated progression towards fibrosis, and poor sustained virological response to interferon-alpha and ribavirin***(Patel et al., 2010)***.The pathogenic association between HCV infection and hepatic steatosis is multifactorial.While hepatic steatosis in patients with HCV infection may be associated with features of the metabolic syndrome through insulin resistance**,** it may be present in the absence of these

findings ***(Eckel et al., 2005)***. 'Viral steatosis' due to direct viral effects through microsomal triglyceride transfer protein, peroxisome proliferators activating receptor and sterol regulatory element binding protein may play a role ***(Patel et al., 2010).***

Both viral proteins and inflammation may lead to reactive oxygen species (ROS) production. HCV core protein increases ROS and lipid peroxidation products through inhibition of electron transport and alterations in permeability of mitochondria which lead to chronic liver injury and fibrosis***(Otani et al., 2005)***. Reducing viral replication has been shown to reduce the quantity of ROS, and increase mitochondrial electron transport activity ***(Ando et al., 2008)***.

Insulin resistance has a key role in the development of hepatic steatosis and potentially steatohepatitis (***Sanyal et al., 2001)***. Resistance to the action of insulin results in important changes in lipid metabolism. These include enhanced peripheral lipolysis, increased triglyceride synthesis, and increased hepatic uptake of fatty acids. Each of these changes may contribute to the accumulation of hepatocellular triglycerides***(Sanyal et al., 2001)***. Excessive fat accumulation in the liverpredisposes it to attack by ROS, leading to lipid peroxidation with its cellular consequences***(Fierbinteanu et al., 2009)***.

Chronic over-production of ROS leads to redox imbalance, favouring depletion ofglutathione (GSH), the major non-enzymatic antioxidant***(Cirioloet al., 1997)***. GSH is involved in the cellular detoxificationof xenobiotics, endogenous toxic metabolites and freeradicals***(Hayes et al., 2005)***. Reduced levels of plasma GSH have been associated with the development of liver fat diseases, cardiovascular disease, diabetes and cancer***(Oniki et al., 2013)***.

The Heme oxygenase (HO) enzyme system comprises three isoenzymes, namely HO-1, 2, and 3, which are encoded by distinct genes ***(Kikuchi et al., 2005)***. While HO-2 and 3 appear to be constitutively expressed, HO-1 is highly inducible not only by its substrate heme but alsounder a variety of conditions associated with oxidative stress ***(Takahashi et al., 2009).*** Recent experimental studies clearly demonstrated that HO-1 expression is a self-defence mechanism against inflammation. It is involved in maintaining cellular homeostasis and many physiological and pathophysiological processes ***(Zhu et al., 2011)***.

It is most likely that many properties including antiinflammation and cytoprotection afforded by HO-1 may be attributed not only its own action but also to other actions of three by-products of HO-1 activity (***Naito et al., 2011)***. HO-1 catalyzes the rate-limiting step in heme degradation to biologically active products: carbon monoxide, biliverdin/bilirubin and controls cellular heme availability. Especially in anti-inflammation, the degradation of the pro-oxidant heme by HO-1 itself, the signalling action of CO, the antioxidant properties of biliverdin/bilirubin, and the sequestration of free iron by ferritin could all concertedly contribute to the anti-inflammatory effects observed with HO-1 (***Ferris et al., 1999; Otterbein et al.,2000; Sedlak & Snyder, 2004;)***.

Since HO-1 is induced as a protective mechanism in response to various stimuli, targeted induction of this stress response enzyme may be considered as an important therapeutic strategy for the protection against inflammatory processes and oxidative tissue damage***(Naito et al., 2011).***HO-1 has been reported to be upregulated in the liver as a result of liver injury due to NAFLD and other liver diseases ***(Malaguarnera et al ., 2005; Yu et al ., 2010; Bessa et al., 2012)***.However, HO-1 is down-regulated in the livers from patients with HCV infection, suggesting that the usual antioxidative HO-1 defence mechanism of the hepatocyte is specifically impaired by the virus causing the hepatocyte to be more prone to oxidative injury ***(Abdalla et al., 2004; Wen et al., 2008)***. To date, the correlation between HO-1 expression and oxidative injury of the liver in HCV and NAFLD patients not clear.

Considering these findings we aimed to highlight the antioxidant potential in NAFLD patients with and without CHC through assessment of the HO-1 gene expression in the liver tissues. We also aimed to study the association between the hepatic HO-1 gene expression andits expression in the peripheral blood, GSH level, liver iron concentration (LIC), HOMA index, and the disease severity to find its relation to disease progression.

2.Patients and methods:

This study was carried out in the National liver Institute, Menufiya University and Internal Medicine department, faculty of medicine, Cairo University in the period from April 2012 to February 2013. A total of 65 patients (40 males and 25 females), age range from 31to 55 yearswere enrolled in the study.Thirty six of them had CHC with NAFLD (24 males and 12 females) and 29 had NAFLD without CHC (16 males and 13 females).

The diagnosis of NASH was established on the basis of the following clinical and histopathological features: (a) abnormal liver biochemistry for more than 3 months; (b) liver biopsy showing steatosis (10%) in the presence of lobular and/or portal inflammation, with or without Mallory bodies or fibrosis; and (c) exclusion of other liver diseases. Diagnosis of chronic hepatitis C was established by serological detection of anti-HCV antibodies and

HCV-RNA, and serum transaminase levels greater than 3 times the upper limit of normal for at least 6 months, and confirmed by liver biopsy.

Exclusion criteria were evidence of liver cirrhosis, malignancy, other causes of liver disease including alcohol ingestion, hepatitis B virus (HBV) infection, autoimmune liver disease, hereditary hemochromatosis, Wilson disease, α-1antitrypsin deficiency, history of intake of drugs known to produce hepatic steatosis (such as corticosteroids ,oestrogen, valproate …) as well as intake of antioxidant vitamin or selenium supplementation within the two months preceding their enrollment in the study .

Additionally 20 (14 males and 6 females) potentially healthy donors to liver transplant withmatched age and sex were included. All controls had normal liver by ultrasound and biochemical liver function tests. They were seronegative for viral hepatitis markers.

Informed written consent was obtained from all participants at the time of their liver biopsy. The study protocol was reviewed and approved by the Ethics Committee of the National Liver Institute-Menoufia University.

Patients and controls were subjected to full history taking and thorough clinical examination. Abdominal ultrasound (US) was done and liver steatosis was assessed semi-quantitatively on a scale of 0 to 3 (0: absent, 1: mild, 2: moderate and 3: severe). Anthropometric measurements were done: Body mass index (BMI) was calculated as weight (in kilograms) divided by height in squared meters. Waist circumference (at the nearest half centimetre) was measured at the midpoint between the lower rib margin and the iliac crest. Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) asfasting glucose (mmol/L) x fasting insulin (μU/ml)/22.5***(Matthews et al., 1985)***. HOMA-IR >3 is considered a criterion of insulin resistance **(*Machado and Cortez-Pinto, 2005*).**

Histological analysis:

Liver biopsies were divided and used for both routine histological examination and determination of HO-1 levels and LIC. ***For histological examination*** sections were fixed and embedded in paraffin blocks and stained with hematoxylin-eosin, Masson's trichrome. The samples were scored according to the NIDDK NASH Clinical Research Network scoring system ***(Kleiner et al., 2005)***. Steatosis was scored from 0 to 3 with four grades scoring system from S0 to S3 (S0: no steatosis or less than 5%, S1: 5%-33%, S2:33%-66%, S3: > 66%). Lobular inflammation was graded as follows:- stage 0: no foci, stage 1: < 2 foci per 200 × field, stage 2: 2-4 foci per 200 × field, stage 3: >4 foci per 200× field. Fibrosis was staged as follows: stage 0: no fibrosis, stage 1: perisinusoidal or periportal fibrosis, stage 2: peri-sinusoidal and portal/periportal fibrosis, stage 3: bridging fibrosis, stage 4: cirrhosis.

Laboratory investigations:

Fasting blood samples were obtained aseptically from patients and controls and divided into 3 parts.One part was collected in a vaccutainer plain tube without anticoagulant to separate serum which is used for determination of liver function tests (serum albumin, aspartate aminotransferase, alanine aminotransferase, gamma glutamyltranspeptidase, alkaline phosphatase), lipid profile including: total cholesterol, low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), triglycerides and fasting blood sugar using COBAS Integra-400 autoanalyzer (Roche-Germany). HBS Ag and anti-HCV antibodies were done on COBAS Amplicore (Roche- Germany). HCV-RNA levels were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) using a commercial kit (Roche Diagnostic, Branchburg, NJ-Germany) according to the manufacturer's instructions.

The determination of fasting insulin level was done using Diagnostic System Laboratories incorporation kits (DSL-10-1600 ACTIVE® insulin, enzyme linked immunosorbent assay (ELISA) kit, Texas- USA). It is an enzymatically amplified one step sandwich type immunoassay. The minimum detection limit is 0.26 uIU/ml.The intra- and inter-assay coefficient variations were 2.6% and 6.2% respectively. Standards, controls and samples were incubated with HRP labelled anti-insulin antibody in microtitration wells which were coated with another anti-insulin antibody, the assay was performed according to the manufacturer's instructions **(*Rasmussen, et al., 1990).***

The second part of sample was drawn into ethylenediaminetetraacetic acid (EDTA) tubes for estimation of GSH. The erythrocyte pellet was washed 3 times with cold isotonic saline and then diluted with saline to the original blood volume. Hemoglobin concentration was determined using a kit supplied by Bio Meriux, France. The erythrocyte-reduced GSH level was measured as previously described **(Beutler, 1963)** and expressed as mmol/g Hb.

The third part was collected in a heparin sterile vacutainer for determination of HO-1 mRNA expression by RT-PCR.

HO-1 determination in peripheral blood by RT-PCR:

Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) using a total RNA

isolation system from Promega Corporation, Madison, USA. RT-PCR reaction for HO mRNA was performed using QIAGEN's 1-step RT-PCR kit (Hilden, Germany), according to the manufacturer's instructions. The final concentrations of the reaction components (50 µL) were 1× QIAGEN buffer, 400 µM of each dNTP, 0.6 µM of both sense and antisense HO primers, 2 µL of RT-PCRenzyme mix, 5 units of RNase inhibitor, and 2 µg of total RNA. Reverse transcription was achieved by heating the reaction components at 50 ºC for 30 min. The initial PCRactivation step was performed by heating at 95 ºC for 15/min.

The amplification reaction was carried out by thermal cycler (model 9600, Perkin-Elmer) for 35 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min, and extension at 72 ºC for 1 min, followed by a final extension step of 72 ºC for 10 min. The HO complementary DNA (cDNA) was amplified using the following primers: sense, 5′-CAGGCAGAGAATGCTGAGTTC-3′ and antisense, 5′-GATGTTGAGCAGGAACGCAGT-3′ (26). ß-actin was used as a housekeeping gene with the following primers: sense, 5′-TTCTTTGCAGCTCCTTCGTTGCCG-3′ and antisense, 5′-TG TGGATGGCTACGTACATGGCTGGG-3′. The amplified product for HO was separated on 2% agarose gel and visualized by ethidium bromide staining under ultraviolet light. Primers for HO were synthesized by Metabion International AG (Martinsried, Germany). The RT-PCR reactions were performed with a Hybaid thermal cycler (Thermo Electron corporation [formerly Hybaid], Waltham, MA,USA). The molecular weight was determined using a DNA ladder and the intensity of PCR bands were analysed by computer program and expressed by arbitrary unit relative to control **(Figure 1).**

HO-1 determination in liver tissue by real-time RT-PCR:

For real-time PCR, liver biopsies were stored in liquid nitrogen immediately after the operation, and kept at -80ºC until RNA extraction. Total RNA from each sample was isolated by use of TRIZOL Reagent (Gibco BRL). Reverse transcription (RT) was performed on 0.8 mg of RNA by use of the Omniscript Reverse Transcriptase kit (QIAGEN), Briefly, 1µg of total RNA, 1µL of oligodT-primer, and 2µL dNTPs were incubated at 65ºC for 5 min, then 10µL of a cDNA synthesis mixture were added and this mixture was incubated at 50ºC for 50min. The reaction was terminated by adding 1µL of RNaseH and incubating the mixture at 37ºC for 20min.

The cDNA was used for HO-1 mRNA quantification by Real-Time RT-PCR technique using the TaqMan system (Perkin Elmer Applied Biosystems, Foster City, CA) as recommended by the manufacturer. Real-Time RT-PCRprimers used for HO-1 mRNA quantification were sense 5′-AGG-GTG-ATA-GAAGAG-GCC-AAG-ACT-3′, (nucleotides 697–720), antisense 5′-TCC-TGC-AAC-TCC-TCA-AAGAGC-3′ (nucleotides 741–761), Real-time RT-PCR was performed as previously described using TaqMan 18S Ribosomal RNA control ***(Brown et al., 2005).***

Relative quantitative analysis was determined by the comparative cycle threshold (Ct) method, which used separate reaction tubes for target (HO-1) and reference (18S rRNA) sequences. Validation experiments showed similar amplification efficiencies for the target gene and the 18S amplicons. The comparative Ct method was used to calculate the amount of target mRNA, normalized to the amount of 18S RNA, and relative to an internal calibrator (control human liver), expressed as 2−ΔDDCt ***(Wen et al., 2008).***

Liver iron concentration (LIC)

LIC was evaluated in liver tissues as described previously. The fresh liver tissue fragment (approximately 15 mm) was weighed and dried at 85ºC for 2 hours in decontaminated quartz vessel and then digested in concentrated nitric oxide (2ml). The resulting solution was then diluted to 5ml with deionized water. Quantitative analysis was performed by inductively coupled plasma-atomic emission spectrometry to determine hepatic iron content. LIC was calculated by dividing LIC in µmol/gm dry weight **(Barry & Sherlock, 1971).**

Statistical analysis

Descriptive statistics were computed for all variables. These included means and standard deviations. ANOVA test was used to compare parametric variables and Kruskal-Wallis test to non parametric variables between the three subject groups. Spearman's correlation coefficients were used to assess associations between HO-1 expression and laboratory and histological characteristics. $P<0.05$ was retained for statistical significance.

3.Results:

Patients and controls demographics and laboratory evaluation

The main demographic and laboratory features of NAFLD and CHC patients are compared with controls in Tables 1 and 2. The control subjects were age and sex matched. BMI, FBS, fasting insulin, HOMA-IR, AST, ALT, alkaline phosphatase and total bilirubin were statistically increased in NAFLD and CHC patients compared to healthy control group. Serum albumin and prothrombin concentration were statistically decreased in CHC patients compared to control subjects. No significant difference was

recorded as regard to these parameters on comparing CHC with NAFLD patients except for serum bilirubin.While GGT and total protein levels were statistically not different among studied groups (Table 2).

Liver iron concentration (LIC)

LIC was significantly elevated in CHC patients compared with control groupand NAFLD patients (Mean±SDwas 53.6±9.2vs 26.4±6.3vs 39.6±7.8 respectively), (P<0.01, P<0.05 respectively). In the later, its level was significantly higher than controls (P<0.05) (Table 3).

Serum GSH level

The serum level of GSH was significantly decreased in CHC (Mean ±SD, 3.04 ± 0.25) and NAFLD patients (Mean±SD,3.98 ± 0.41) compared to control group (Mean±SD, 5.63 ±0.27), (P<0.01 and P<.0.05 respectively). Its level was not statistically different among both patient groups (P>0.05) (Table 3 & Figure 2).

HO-1 mRNA expression in the liver and its correlation with HO-1 expression in the peripheral blood, LIC, GSH levels, HOMA index, NAFLD grade and fibrosis stage:

HO-1 mRNA expression in the liver biopsies of NAFLD patients (Mean ±SD, 1.98±0.31) was higher than that of the controls (Mean ±SD, 1.16±0.15)and the difference was statistically significant(p<0.01). In contrast CHC patients showed significantly lower HO-1 mRNA expression (Mean ±SD, 0.67±0.22) than the controls (p<0.01). On comparing the levels of expression in both patient groups, significantly higher levels were observed in NAFLD patients (p<0.001) (Table 3& Figure 3).

In the peripheral blood we observed corresponding pattern of HO-1 mRNA expression as that of the liver tissues in the studied group where the peripheral blood HO-1 mRNA expression of NAFLD patients (Mean ±SD, 173.6 ± 18.52) was higher than that of the controls (Mean ±SD, 116.53 ± 10.32)and the difference was statistically significant(p<0.01). In contrast CHC patients showed significantly less HO-1 mRNA expression (Mean ±SD, 72.46 ± 28.34) than the controls (p<0.01). On comparing the levels of expression in both patient groups,significantly higher levels was observed in NAFLD patients (p<0.001) (Table 3 & Figure 4).

In CHC patients negative association was observed between the level of hepatic HO-1 mRNA expression and the peripheral blood HO-1 mRNA, HOMA index and LIC (r = -0.41, -0.44, -0.46 ; p<0.05). Also a negative association was observed with NAFLD grade and fibrosis degree (r = -0.61, -0.68; p<0.01), whereas its level was positively correlated with the GSH level (r = 0.57; p< 0.01) (Table 4).

On the other hand, in NAFLD patients positive associations were observed between the level of hepatic HO-1 mRNA expression and peripheral blood HO-1 mRNA, HOMA index and LIC (r = 0.49, 0.56, 0.42, p< 0.05, <0.01, < 0.05 respectively). Also a positive association was observed with NAFLD grade and fibrosis degree (r = 0.72, 0.51; p<0.001 and <0.05, respectively). The correlation between the hepatic HO-1 expression and GSH levels was negative (r = -0.61; p< 0.01) (Table 4).

Table (1) Baseline characteristics of the subjects studied

Parameters(mean±SD)	**CHC** (N=36)	**NAFLD** (N=29)	**Controls** (N=20)	***p*-value**
Age (years)	48.2±6.3	46.7±6.4	38.7±4.8	NS
Gender (male %)	24 (66.7%)	14 (48.3%)	14 (70%)	NS
BMI (kg/m^2)	30.6±1.5	31.2±1.3	24.2±1.7	P< 0.05
Triglyc. (mg/dl)	145.7±11.2	148.3±13.9	102.4±16.5	P< 0.05
T-cholesterol	198.1±19.5	194.5±16.5	165.4±13.6	P< 0.05
HDL-c (mg/dl)	32.4±4.1	33.5±4.0	36.9±4.2	P< 0.05
LDL-c (mg/dl)	103.7±9.2	118.9±15.4	96.2±8.5	P< 0.05
Fasting blood sugar	98.6±8.2	101.3±11.5	78.5±6.9	P< 0.05
Fasting Insulin (uIU/ml)	7.3±2.2	8.9±2.0	6.8±0.7	P< 0.05
HOMA-IR	2.76±1.02	3.34±1.12	2.30±0.77	P < 0.05
Grade of steatosis (No %):				
Mild (grade 1)	11 (30.56%)	12 (41.38%)		
Moderate (grade 2)	15 (41.66%)	13 (44.83%)		
Severe (grade 3)	10 (27.78%)	4 (13.79%)		

(***p***<0.05 is statistically significant)

Table (2) Liver function tests among studied groups.

Parameters(mean±SD)	**CHC (N=36)**	**NAFLD (n=29)**	**Controls (n=20)**	***p*-value**
AST (U/L)	52.4±8.1	40.2±9.1	22.0±8.7	P^1<0.01;P^2<0.05;P^3>0.05
ALT (U/L)	62.4±12.6	51.6±14.6	20.1±5.6	P^1<0.01; P^2<0.05;P^3>0.05
Alk. Ph. (U/L)	48.2±10.2	56.8±11.2	31.2±7.6	P^1<0.05; P^2<0.05; P^3>0.05
GGT (U/L)	36.4±7.8	45.6±10.2	33.2±5.4	P^1>0.05; P^2>0.05; P^3>0.05
S.blirubin (mg/dl)	3.7±1.93	1.9±0.34	0.97±0.41	P^1<0.001; P^2<0.05;P^3<0.05
T. protein (g/dl)	7.2±1.32	7.5±1.02	7.9±0.87	P^1>0.05; P^2>0.05; P^3>0.05
S. albumin (g/dl)	3.87±0.45	4.03±0.35	4.71±0.33	P^1<0.05; P^2>0.05; P^3>0.05
Prothrombin con.%	76.3±5.7	81.3±4.7	95.7±3.2	P^1<0.05; P^2>0.05; P^3>0.05

P^1:CHCvs control, P^2:NAFLD vscontrol, P^3: CHC vs NAFLD. (p<0.05 is statistically significant)

Table (3) LIC, GSH andHO-1 mRNA expression in studied groups

Parameters(mean±SD)	**CHC** (n=36)	**NAFLD** (n=29)	**Controls** (n=20)	***p*-value**
LIC (µmol/gm dry weight)	53.6±9.2	39.6±7.8	26.4±6.3	P^1<0.01;P^2<0.05;P^3<0.05
GSH (µmol/g Hb)	3.04 ± 0.25	3.98 ± 0.41	5.63 ± 0.27	P^1<0.01;P^2<0.05P^3>0.05
Peripheral blood HO-1 mRNA (arbitrary units)	72.46 ± 28.34	173.6 ± 18.52	116.53 ± 10.32	P^1<0.01;P^2<0.01P^3<0.001
Liver HO-1 mRNA (arbitrary units)	0.67±0.22	1.98±0.31	1.16±0.15	P^1<0.01;P^2<0.01;P^3<0.001

P^1:CHC vs control, P^2: NAFLD vs control, P^3: CHC vs NAFLD. (p<0.05 is statistically significant)

Table (4) Correlation between Liver HO-1 expression and some parameters in studied patient groups

Parameters	**CHC associated NAFLD (N =36)**		**NAFLD (N=29)**	
	***r*-value**	***P* -value**	***r*-value**	***P*-value**
Peripheral blood HO-1 mRNA	- 0.41	<0.05	0.49	<0.05
GSH levels	0.57	<0.01	- 0.61	<0.01
HOMA index	- 0.44	<0.05	0.56	<0.01
LIC	- 0.46	<0.05	0.42	<0.05
NAFLD grade	- 0.61	<0.01	0.72	<0.001
Fibrosis degree	-0.68	<0.01	0.51	<0.05

(*p*<0.05 is statistically significant)

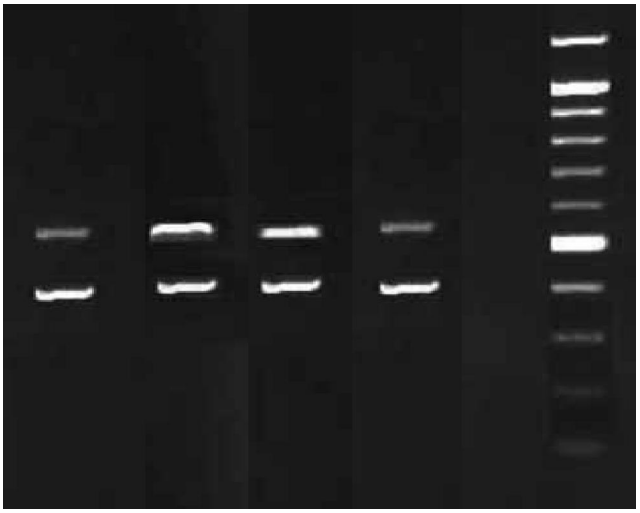

Figure (1) Shows PCR product of HO-1 expression in peripheral blood, lane M=1000 bp ladder, lane 1 =CHC, 2 & 3 NAFLD, lane 4= control, lane 5= blank.

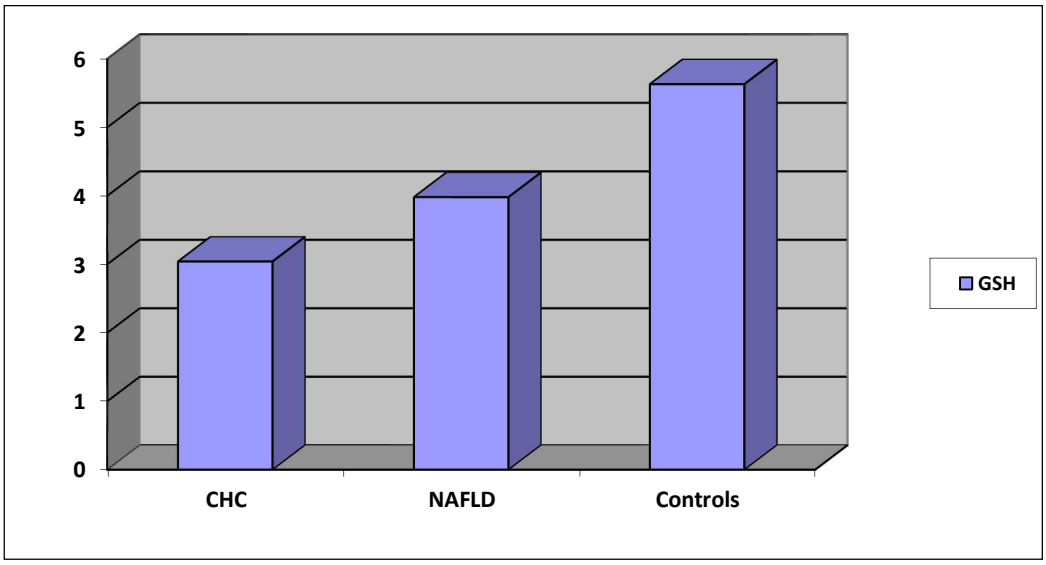

Figure (2) Serum levels of GSH among 3 studied groups.

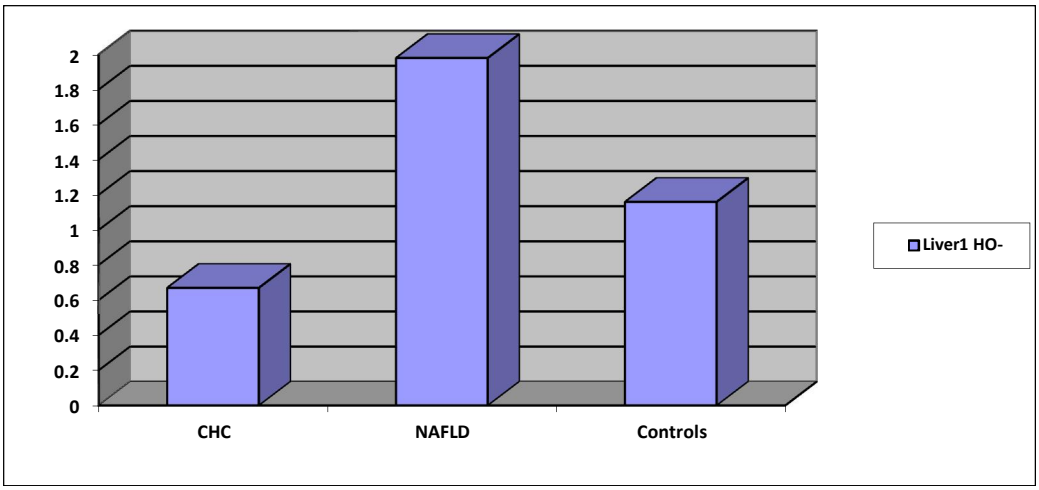

Figure (3) Level of HO-1 expressions in the livers of three studied groups.

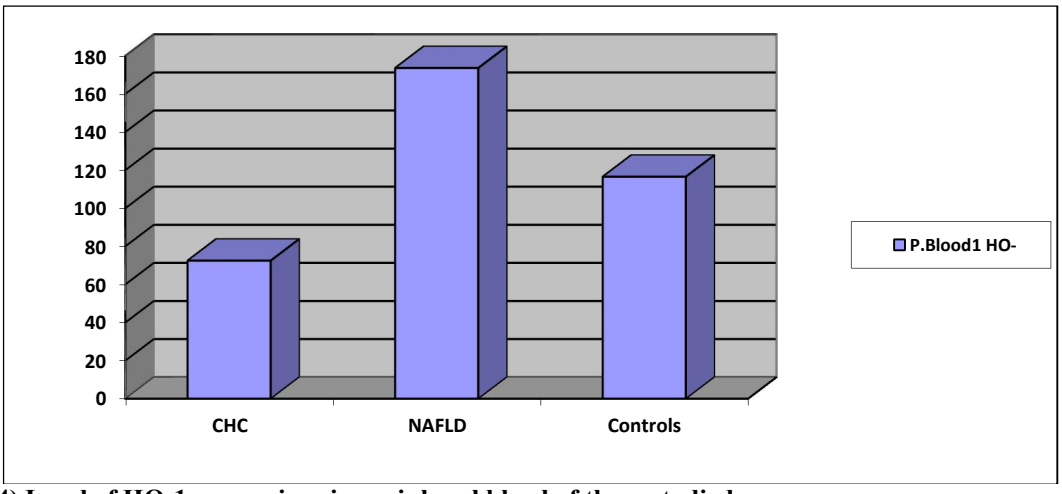

Figure (4) Level of HO-1 expressions in peripheral blood of three studied groups.

4.Discussion:

Non-alcoholic fatty liver disease (NAFLD), the hepatic counterpart of the metabolic syndrome, encompasses a disease spectrum spanning steatosis through non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma(HCC)***(Vanni et al., 2010)***.A key step in the development of both hepatic steatosis and hepatic injury is oxidative stress. An imbalance of reactive oxygen species (ROS) and antioxidants, such that the pro-oxidant activity exerted on different molecules are potentially able to alter their structure and function***(Sofieet al., 2013)***.The underlying causes of oxidative stress during chronic HCV infection are not fully understood; however the cellular expression of individual hepatitis C viral proteins, such as HCV core and NS5A, increases the cellular generation of ROS***(Fierbinteanu-Braticevici et al., 2009).***

Heme oxygenase-1 (HO-1) is an oxidative stress-response gene up-regulated by various physiological and exogenous stimuli ***(Zeng et al., 2011)***. It has been shown to display cytoprotective properties, as well as anti-inflammatory, antioxidant, antiapoptotic, and possible immunomodulatory activities ***(Kirkby & Adin, 2006; Nath, 2006; Ryter & Choi, 2009)***. The importance of HO-1 in protection against various organs diseases has been reported (***Lakkistoet al., 2011; Yang et al., 2009; Yu et al., 2010 – Bessaet al., 2012).*** In the current study we assessed the level of HO-1 gene expression in the liver tissues in NAFLD patients with and without HCV in an attempt to shed light on its antioxidant potential and whether it has any relation to the disease severity and progression.

We demonstrated that the expression of HO-1 mRNA in the liver biopsies of NAFLD patients was significantly higher than that of the controls.Patients with CHC showed significantly lower HO-1 mRNA expression than the controls. On comparing the level of expression in both patient groups,significantly higher levels were observed in NAFLD patients. Same pattern of HO-1 mRNA expression was observed in the peripheral blood in the studied group. Also we found a positive association between HO-1 mRNA in the liver biopsies and the disease severity in NAFLD patients.On the other hand a negative association was demonstrated in CHC patients.

In concordance with our results; others have demonstrated that HO-1 expression was significantly increased in NASH patients, and this increase reflected the severity of the disease (***Malaguarneraet al., 2005; Abdalla et al., 2012;Bessa et al., 2012;)***. ***Wen et al.(2008)*** highlighted the link between HO-1 and HCV. Their findings showed that expression of HCV core protein leads to increased sensitivity of cells to cytotoxins and other cellular stressors. These events are accompanied by increased levels of prooxidants and attenuation of the induction of HO-1 expression that normally occurs in response to stress.Similar findings were described recently by***Abdalla et al.(2012)***whoshowed thatHO-1 expression was upregulated in response to NAFLD; however when HCV is present with NAFLD, HO-1 expression is suppressed. Despite NAFLD upregulates the HO-1 enzyme, it may perhaps be the key in reducing its expression in HCV patients ***(Abdalla et al., 2012).***These results support their previous study demonstrating that HCV down regulates HO-1 in the liver as compared to controls (***Abdalla et al., 2004).***

On the contrary to our results, ***Bessaet al.(2012)*** observed that HO-1 mRNA expression was increased in both patients with NASH and those with CHC compared to controls. This contradiction may probably be because they didn't specify whether CHC in their patients was associated with NAFLD or not.Also it may be due to the limited number of studied patients and different method of measuring HO-1 mRNA which could beactually decreased in core-expressing cells because of decreased cellular viability due to cytotoxin (***Wen et al., 2008***).

GSH is a well-established, metabolic regulator that may qualify as a putative index of health. In humans GSH depletion is linked to a number of disease states.There is a growing awareness about this molecule's role in the pathophysiology of many diseases as a non-enzymatic antioxidant ***(Chavan et al., 2005).***

In the current study we demonstrated a significant decrease in the level of GSH antioxidant in CHC and NAFLD patients compared to controls. Its level was not statistically different among both patient groups. In CHC a positive association has been observed between HO-1 expression and GSH while a negative association was observed in NAFLD patients.

Mitochondrial abnormality may play a role in the onset and progression of NASH in correlation with oxidative stress ***(Kojima et al., 2007).***Different HCV protein was shown to bind to mitochondria and affect mitochondrial depolarization leading to depletion of reduced glutathione ***(Otani et al., 2005).*** Core protein-expressing cells also exhibited increased levels of superoxide and peroxides, which may be a consequence of depleted glutathione and thioredoxin stores (***Wen et al., 2004; Abdalla et al., 2005; Otani et al., 2005;).***

Malaguarnera(2005) and his colleague, demonstrated that NASH patients with lower levels of GSH exhibited higher expression of HO-1, which is consistent with our study.HO-1 induction is an adaptive response against oxidative damage elicited

by lipid peroxidation and it may be critical in the progression of the disease. Failure of HO-1 upregulation in our patient group with concomitant HCV infection despite of the lower level of GSH could be through direct or indirect interaction of HCV different proteins in the HO-1 induction pathway ***(Abdalla et al., 2012)*.**

Reactive oxygen stress (ROS) have numerous effects in the liver, induction of cytokine formation by hepatocytes and Kupffer cells leads to increased TNF-a production and insulin resistance, while development of insulin resistance may result in hepatic steatosis. ROS may cause genetic mutations and chromosomal alterations contributing to carcinogenesis ***(Koike & Moriya,2005).***

The relationship between HO-1 and insulin sensitivity had been shown by***Nicolai et al.(2009).*** They demonstrated that HO-1 induction improved insulin sensitivity and caused adipose tissue remodelling in a model of obesity-induced insulin resistance suggesting that HO-1 may be involved in the pathogenesis of insulin resistance syndrome. Furthermore, others reported on the protective effects of HO-1 upregulation on the function and survival of insulin producing β-cells. Strategies aimed at HO-1 upregulation might result in better outcome in islet transplantation ***(Pileggi et al., 2001).***In this studywe found a positive associationbetween the level of hepatic HO-1 mRNA expression in NAFLD and HOMA index while in CHC a negative association was detected

Elevated iron stores had been found to facilitate oxidative stress, steatosis, hepatocarcinogenesis and also affect the outcome of antiviral therapy ***(Valentet al., 2007;Furutani et al., 2006)***. Previous study had shown that HO-1–deficient mice develop severe iron deposition in the liver and kidney and exhibit macromolecular oxidative damage, tissue injury, and chronic inflammation ***(Poss & Tonegawa, 1997).*** Genetic deficiency of HO-1 in humans exhibited the same effects (***Yachi, 1999).*** We observed that LIC was significantly elevated in CHC patients compared with healthy controls and NAFLD patients. In the later, their level was significantly higher than controls. We demonstrated a positive association between LIC and the level of HO-1 expression in NAFLD patients while a negative association was observed with CHC patients.

Although the underlying mechanism by which HO-1 facilitates the iron metabolism is unclear ***(Juan et al., 2001)***,it had been shown that intracellular iron accumulation was reduced by HO-1 over expression in experimental study through augmentation of iron efflux from cells ***(Ferris et al.,1999; Juan et al., 2001).***

Despite the mentioned protective role of HO-1 (***Kirkby & Adin, 2006; Nath, 2006; Ryter & Choi, 2009;)***, others urging that HO-1 downregulation in certaindiseases could protect against the potential toxic consequences of increased cellular free iron ***(Suttner & Dennery, 1999).Malaguarnera et al., (*2005)** underlined the potential pro-oxidant consequences of HO activity. It is the balance of heme and reactive iron which determined the antioxidant effect of HO-1. At low HO-1 expression, low cellular heme and low iron may allow for decreased oxidative injury and upregulation of important enzymes, whereas excessive accumulation of reactive iron at high HO-1 expression would result in increased oxidative stress, cytotoxicity, and abnormal cellular proliferation (***Suttner & Dennery, 1999).***

From the present study, it is recognized that steatohepatitis is associated with oxidative stress, as evidenced by increased LIC level with decrease in GSH level. The body defence against it could be the determinant for disease progression. In HCV patients the reduced level of HO-1 possibly will be an important correctable factor. It is presently unclear how regulation of HO-1 expression bangs on the disease outcome. Further study is needed on large sample size for better understanding of the functional outcome achieved by upregulation of HO-1expression taken into consideration its protective antioxidant effect and possible pro-oxidant activity considering propermodulation of the HO-1 pathway which may provide a new therapeutic approach to arrest further disease evolution.

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