Tumor Necrosis Factor- α and Iron Overload Are Associated with Insulin Resistance in Hepatitis C in Egyptian Patients

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Abstract

Background: There are evidences of an increased incidence of insulin resistance (IR) in patients with hepatitis C virus infection. Several mechanisms have been proposed to explain the increased IR in hepatitis C.

Aims: To evaluate tumor necrosis factor-alpha (TNF- α) and iron overload as potential mediators of IR development in chronic HCV patients.

Patients and Methods: Study groups consisted of four groups: patients with HCV infection (n = 35), patients with hepatitis C and diabetes mellitus (HCV+DM) (n = 31), patients with DM (n = 36), and healthy controls (n = 30). The homeostatic model assessment (HOMA) was the criteria used to quantify the degree of IR.

Results: HOMA indices were significantly higher in the HCV, HCV+DM and DM groups compared to the healthy controls ($P < 0.001$ for each). TNF- α was significantly higher in HCV and HCV+DM groups compared with DM and healthy controls ($P < 0.001$ for each). TNF- α was significantly higher in HCV+DM group compared with HCV group ($P < 0.05$). Furthermore, TNF- α was positively correlated with HOMA indices in HCV and HCV+DM groups ($r = \sim +0.900$, $P < 0.001$). Serum ferritin had a positive correlation with HOMA indices in HCV and HCV+DM groups ($r = \sim +0.800$, $P < 0.001$).

Conclusion: TNF- α and iron overload may explain in part the development of IR in chronic HCV patients.

Keywords: diabetes; ferritin; transferrin.

1. Introduction

Hepatitis C virus (HCV) infection is a common problem affecting over 3% of the world's population [1]. Egypt has a high prevalence of HCV infection and many patients of them develop chronic hepatitis C (CHC) [2]. CHC accounts for 50%-76% of all liver cancer cases worldwide, and has been associated with significant morbidity and mortality rates [1,3]

Furthermore, CHC is associated with a high prevalence of type 2 diabetes (T2DM) [4,5]. Development of T2DM in CHC predicts faster rate of liver fibrosis and cirrhosis with a poor response to antiviral therapy [6]. Although several mechanisms have been postulated, including insulin resistance (IR) and inadequate insulin secretion [7], however, thus far, the mechanisms underlying in T2DM in HCV infections are not well understood.

The prevalence of T2DM in patients with cirrhosis due to HCV is more than that of cirrhosis from other causes; also, T2DM is associated with CHC even in the absence of cirrhosis [8]. There was no association between CHC and type 1 diabetes, or with chronic hepatitis B (CHB) [9,10]. A report by Mehta et al. [11] has demonstrated that HCV-associated T2DM mainly occurs in patients with other risk factors, such as, greater body mass index (BMI) and older age. Thus, among patients classified as "high risk" for T2DM, CHC increased diabetes risk more than 11-fold [11].

Moucari et al. [9] have also shown that IR is increased in patients infected with HCV however, it is not necessary that all patients with CHC develop IR. This report suggests a complex interaction between the virus and other host factors. Several authors have reported a reduction in HOMA indices in patients with HCV following treatment, and an association between IR and HCV following decreased viral loads [6,9]. Moucari et al. [9] have concluded that the effects of HCV genotype on insulin signaling are not well understood.

Previously, Tam et al. [12] have suggested that, chronic inflammation plays a significant role

in the development of IR associated with liver disease, due to increased levels of interleukin-1 (IL-1), IL-6 and TNF- α [12]. Some researchers [13,14] are of the opinion that IR occurs in other inflammatory conditions, such as, inflammatory bowel disease and rheumatoid arthritis following elevation in the levels TNF- α . Initial studies in this context have noticed increased serum levels of TNF- α in CHC [4,15]. TNF- α can induce IR by direct and indirect mechanisms. TNF- α can inhibit the tyrosine phosphorylation of the insulin receptors and insulin receptor substrate-1 (IRS-1) [16]. Furthermore, TNF- α also directly reduces the β cells function [17].

The relationship between iron overload and the development of T2DM is well established [18]. There is a positive correlation between body iron stores and CHC [19]. Iron overload may contribute to the development of IR and DM in chronic HCV patients [18].

This study is designed to investigate the association between CHC and IR in Egyptian patients and their correlation with TNF- α and iron homeostasis as co-morbid factors in the development of IR in CHC in comparison with age and sex matched control.

2. Methods

This study was conducted on 102 patients who were attending the outpatient clinic in the National Liver Institute, Menoufiya, Egypt, as well as healthy volunteers who served as healthy control group. The subjects were divided into 4 groups: healthy control (C) (n=30), diabetes type 2 (DM) (n= 36), chronic HCV (HCV) (n= 35), and chronic HCV with DM (HCV+DM) (n=31).

All participants were subjected to history taking and clinical examination. Exclusion criteria among the participants was, hepatitis B infection, history of alcohol consumption, pregnancy, acute infection, previous treatment with interferon [because interferon can produce a transient increase of IR [20], treatment with any other medication known to affect glucose tolerance or insulin secretion e.g. corticosteroids, or the presence of other concomitant diseases such as hemochromatosis, or other serious medical problems. Abdominal ultrasound was done for all

the participants to assess the liver, portal vein diameter, spleen size and presence or absence of ascites. An informed consent was obtained from each patient included in the study. The study was conducted in accordance with the ethical guidelines and was approved by the ethical committee of the National Liver Institute, Menoufiya University, Egypt.

All groups were matched by age, sex, BMI, waist-to-hip ratio. After an overnight fasting, venous blood was collected. Plasma glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, prothrombin concentration percentage (PT%), glycated hemoglobin (HbA_{1c}) and triglyceride levels were determined by standard laboratory techniques used in clinical chemistry laboratories (Abbott Laboratories, Chicago IL). Schistosomal antigen was done to exclude the active schistosomal infection. Serological markers for HbsAg, anti-HbcAb, Anti-HCV were measured by using enzyme immunoassay (Abbott Laboratories, Chicago IL). All anti-HCV⁺ patients were HCV-RNA⁺ as confirmed by HCV-RNA qualitative testing (Amplicor; Roche Molecular Systems, Branchburg, NJ). Serum iron, transferrin and total iron binding capacity (TIBC) were measured using commercial kit (Roche, USA) [21]. Serum ferritin was measured by a particle-enhanced immunoturbidimetric assay using the Hitachi 911 analyzer (Roche Diagnostics, Basle, Switzerland).

Serum insulin was determined by enzyme amplified sensitivity immunoassay performed on microtiterplates which has a sensitivity of 6.4 pmol/L (BioSource Europe, Nivelles, Belgium). Serum TNF- α concentration was measured by enzyme-linked immunosorbent assay with a detection limit of 0.5pg/mL (Quantikine; R&D Systems Europe, Abingdon Lane, U.K.). While the euglycemic hyperinsulinemic clamp is the standard method for measuring insulin resistance, a common method using the homeostasis model HOMA of IR was calculated by using the following equation: $\text{HOMA} = \text{fasting glucose (mmol/L)} \times \text{fasting insulin (mU/L)} / 22.5$. Typically, a HOMA value > 2 is used to identify significant IR [22].

3. Statistical analysis

Results were expressed as the mean $\pm$ SD or as median (range). ANOVA test was used for comparison among different groups in quantitative data. All *P* values are based on a two-tailed test of statistical significance, which was accepted at the level of *P* < 0.05. Linear correlation coefficient was used for detection of correlation between two quantitative variables. Statistical analyses were performed with the SPSS statistical package for windows.

4. Results

The clinical and biochemical features of patients included in the study are shown in table 1. Patients with DM had a significantly higher BMI (31.4 ± 2.8) compared to the control group (25.1 ± 1.2) (*p* < 0.05), while there was no significant difference in BMI between patients with HCV (25.8 ± 1.1) or HCV+DM (26.5 ± 1.7) and the control group. Patients in HCV and HCV+DM groups had a significantly higher ALT and AST levels compared to the control and DM groups (*p* < 0.001).

Data presented as mean $\pm$ SD or *n* (%). DM: diabetes mellitus; HCV: hepatitis C virus; BMI: body mass index; FBG: fasting blood glucose; ALB: albumin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; PT%: prothrombin concentration percentage; TIBC: total iron binding capacity, * = significant difference versus the control group (*p* < 0.01).

Patients in HCV (146.1 ± 50.4) and HCV+DM (517.2 ± 98.9) groups had a significantly higher serum level of ferritin (ng/ml) in comparison to patients with DM (86.9 ± 63.4) and the control subjects (76.7 ± 55.2) (*p* < 0.001). However, there was no significant difference in serum ferritin between HCV patients and patients with HCV+DM (Table 1). Transferrin saturation (%) in HCV (52.3 ± 8.5) and HCV+DM (69.2 ± 7.9) groups were significantly higher (*p* < 0.01 for each) in comparison to patients with DM (25.1 ± 6.2) and the control subjects (28.1 ± 4.5).

Serum levels of TNF- α (pg/ml) were significantly higher in HCV group compared with controls (16.9 ± 2.7 and 2.9 ± 2.1 , respectively; *P*

< 0.001) (figure 1). The present study also, showed a significant increase in TNF- α levels in HCV+DM group (24.9 ± 3.6) when compared with DM (4.6 ± 1.3) and control groups ($P <$

0.001) (Figure 1). There was no significant difference between patients with HCV and those with HCV+DM regarding the serum TNF- α .

Table 1. Basic clinical and biochemical parameters of all groups

	Control (n=30)	DM (n=36)	HCV (n=35)	HCV+DM (n=31)
Age (yr)	40.7 $\pm$ 5.7	44.6 $\pm$ 6.9	40.2 $\pm$ 6.8	42.5 $\pm$ 5.9
Female (n)	12	14	11	12
BMI (kg/m²)	25.1 $\pm$ 1.2	31.4 $\pm$ 2.8	25.8 $\pm$ 1.1	26.5 $\pm$ 1.7
FBG (mmol/l)	4.42 $\pm$ 0.59	9.82 $\pm$ 0.78 *	5.16 $\pm$ 0.49	8.56 $\pm$ 1.28 *
Bilirubin (mg/dl)	0.52 $\pm$ 0.3	0.48 $\pm$ 0.2	0.65 $\pm$ 0.3	0.68 $\pm$ 0.2
ALB (g/l)	4.7 $\pm$ 0.2	4.3 $\pm$ 0.3	3.9 $\pm$ 0.5	4.1 $\pm$ 0.7
ALT (U/l)	18.8 $\pm$ 5.6	22.5 $\pm$ 7.3	50.4 $\pm$ 28.5 *	72.8 $\pm$ 68.8 *
AST (U/l)	20.9 $\pm$ 5.5	23.2 $\pm$ 6.4	47.1 $\pm$ 19.3 *	80.7 $\pm$ 59.6 *
PT(%)	92.8 $\pm$ 12.8	91.5 $\pm$ 8.7	87.5 $\pm$ 14.8	88.6 $\pm$ 17.9
Iron (μmol/l)	17.8 $\pm$ 5.3	16.3 $\pm$ 4.1	26.5 $\pm$ 6.7	31.7 $\pm$ 7.2 *
Ferittin (μg/l)	76.7 $\pm$ 55.2	86.9 $\pm$ 63.4	146.1 $\pm$ 50.4 *	517.2 $\pm$ 98.9 *
Transferrin (g/l)	2.78 $\pm$ 0.27	2.53 $\pm$ 0.32	2.41 $\pm$ 0.25	1.89 $\pm$ 0.21 *
TIBC (μmol/l)	63.3 $\pm$ 8.41	65.1 $\pm$ 7.63	51.3 $\pm$ 14.3	46.1 $\pm$ 7.12 *
Transferrin saturation (%)	28.1 $\pm$ 4.5	25.1 $\pm$ 6.2	52.3 $\pm$ 8.5 *	69.2 $\pm$ 7.9 *
Insulin (mU/l)	6.2 $\pm$ 2.1	11.2 $\pm$ 5.4 *	17.5 $\pm$ 7.2 *	14.9 $\pm$ 5.7 *

Figure 1. Levels of serum tumor necrosis factor alpha (TNF- α) in the control group, patients with diabetes mellitus (DM), patients with HCV infection (HCV), and patients with HCV infection and diabetes mellitus (HCV+DM). Results are expressed as mean $\pm$ SD. a = significant difference versus the control group ($p < 0.001$), b = significant difference versus the HCV group ($p < 0.05$).

Figure 2. Homeostatic Model Assessment (HOMA) index values in the control group, patients with diabetes mellitus (DM), patients with HCV infection (HCV), and patients with HCV infection and diabetes mellitus (HCV+DM). Results are expressed as mean $\pm$ SD. a = significant difference versus the control group ($p < 0.001$) and b = significant difference versus DM and HCV groups ($p < 0.01$).

Table 2. Pearson correlation coefficients between serum ferritin and TNF- α with AST, ALT and HOMA

		TNF- α		Ferritin	
		r	P	r	P
HCV					
ALT		0.979	<0.001	0.755	<0.001
AST		0.524	0.023	0.354	0.055
HOMA		0.904	<0.001	0.889	<0.001
HCV+DM					
ALT		0.774	<0.001	0.858	<0.001
AST		0.410	0.025	0.506	<0.01
HOMA		0.982	<0.001	0.874	<0.001

HCV: patients with hepatitis C virus; HCV+DM: patients with HCV infection and diabetes mellitus; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TNF- α : tumor necrosis factor-alpha; HOMA: homeostatic model assessment.

The present study revealed a significant increase in the fasting insulin level (mU/l) in both

HCV (17.5 ± 7.2) and HCV+DM (14.9 ± 5.7) patients in comparison with the control group (6.2

± 2.1) ($p < 0.01$) (Table 1). HOMA index values in group DM (3.69 ± 0.2), HCV (4.4 ± 0.2) and HCV+DM (6.1 ± 0.32) were significantly higher ($p < 0.01$) than in control group (1.3 ± 0.21) (figure 2). Furthermore, patients with HCV+DM had a significantly higher HOMA index values compared to those of DM alone ($p < 0.05$).

Furthermore, serum levels of TNF- α was positively correlated to HOMA indices in HCV ($r=0.904$, $p < 0.001$) and HCV+DM ($r=0.982$, $p < 0.001$) groups (Table 2). Serum TNF- α level had a positive correlation with ALT in HCV ($r=0.979$, $p < 0.001$) and HCV+DM ($r=0.774$, $p < 0.001$) groups (Table 2). Serum ferritin levels also had a positive correlation with HOMA indices in HCV ($r=0.889$, $p < 0.001$) and HCV+DM ($r=0.874$, $p < 0.001$) groups. Serum ferritin level had a positive correlation with ALT in HCV ($r=0.775$, $p < 0.001$) and HCV+DM ($r=0.858$, $p < 0.001$) groups and with AST in HCV+DM ($r=0.506$, $p < 0.01$) group.

5. Discussion

Both HCV and DM are common diseases in Egypt although they seem to share little in common but their concurrent presence is higher than would be expected [23]. Therefore, our investigation is of significant importance in finding out the association between HCV and DM for several reasons. First, DM adversely affects the HCV patients and is associated with increased liver steatosis and fibrosis [24]. Secondly, HCV patients associated with DM are less responsive to interferon therapy [25] and showed an increased prevalence of liver cancer [26].

Previously, it has been demonstrated that, IR results from defects in the insulin signaling pathway, including defects in the insulin receptor or IRS molecules [27]. These defects can result from either reduced levels of signaling proteins, or modulation of their activity by phosphorylation [28]. The present study showed increase fasting insulin and HOMA indices in HCV patient, which indicating the development of IR. These results are in consistence with other studies which reported that IR is a specific feature of hepatitis C and present in about 35% of patients with CHC but not CHB [9,10]. Also, HCV-infected patients who respond to antiviral therapy show improved

insulin sensitivity [29,30]. Ishida et al. [31] have speculated that the possible mechanism for the development of IR in HCV patients is through p21-activated kinase 1, which is stimulated by PI3K/Akt. HCV decreases the PI3K/Akt signaling to favor the viral replication [31].

Other researchers [32,33] have proposed another possible mechanism, that might have contributed to the development of IR in HCV patients, is the increase in TNF- α level. Our results are in congruence with these studies [32,33]. There are some evidences supporting the relation between TNF- α and the development of IR and T2DM [34]. It has been shown that TNF- α receptors were increased in HCV+DM patients compared with HCV and controls [4]. Moreover, the clearance of HCV with subsequent reduction of serum TNF- α induces an improvement in IR [6]. Another study in transgenic mice revealed that expression of HCV core protein induces hepatic IR with elevated circulating levels of TNF- α . These levels are reversed by TNF- α antibody [35]. Furthermore, it is also reported that TNF- α could interfere with insulin signaling by inhibiting the tyrosine kinase activities of the insulin receptors [17]. TNF- α could also stimulate I κ B kinase β (IKK β) which inactivates the insulin signaling molecule IRS-1 [36]. Thus, it is likely that TNF- α is not the only factor for development of IR in CHC but other virus-specific factors are also involved which affect the insulin signaling.

Ferritin is not only a marker of iron load but is also increased in inflammatory states. Serum ferritin is associated with an increased risk of IR and DM [37] and was found to be significantly higher in T2DM than normal subjects [20,38]. The present study showed increases in serum iron and ferritin level in HCV patients compared with non diabetic HCV patient. Iron overload has been found to reduce the hepatic metabolism of insulin leading to hyperinsulinemia [39]. This process can lead to redistribution of transferrin receptors on the cell surface and stimulation of more iron uptake by the liver [40]. Other investigators have found that only HCV patients with concomitant DM have high levels of serum ferritin in comparison to HCV patients without DM [41]. It could be argued that ferritin is an independent predictor of an increase in fasting insulin,

development of IR independently of TNF, suggesting that the iron status itself may be involved

The mechanisms of IR development in HCV patients with increased serum ferritin level may also, include the inflammatory process in HCV patient and the iron oxidative stress [42]. It has been hypothesized that the formation of hydroxyl radicals catalyzed by iron contributes to IR with subsequent development of T2DM [43]. Other studies support the hypothesis of elevated iron stores as an etiological factor in IR rather than in β -cell dysfunction [44]. The HCV itself has a role in the increased oxidative stress. In *in vitro* studies have demonstrated increased mitochondrial reactive oxygen stress in hepatoma cells and concomitant over-expression of core protein and in HCV in core transgenic mice [45]. Oxidative stress, in HCV-infected patients, has been shown to correlate with IR, independent of obesity [46]. It has been documented that oxidative stress and IR contribute to steatosis, which in turn exacerbates both IR and oxidative stress [47]. This may imply a role for antioxidants as a protective therapy [48]. However, the interaction between oxidative stress and IR is more complex and need further researches.

In our study we observed a positive correlation between plasma transferrin concentrations, fasting insulin, and TNF- α . This observation may be explained, at least in part, by the previously reported which suggests up regulation of transferrin production by insulin in human hepatocytes [49]. Transferrin may be involved in the pathogenesis of IR through its antagonist effect on insulin action [50] and through its enhancing effect on the rate of the lipolysis in adipocytes [51] which increases the availability of free fatty acids to the liver and skeletal muscle, which could in turn lead to IR [52]. Also, studies on blood donors indicated that depletion of iron stores may prevent the development of IR [53]. In summary, both ferritin and transferrin levels were associated with hyperinsulinemia and hyperglycemia. Serum ferritin and transferrin could be independently predictive of development of IR or T2DM in CHC.

Our findings are suggesting a potential role for iron metabolism and TNF- α , at least in part, in explaining the higher prevalence of IR and T2DM in CHC patient. Further studies are needed to evaluate the molecular mechanisms which are involved.

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