The Role of Myeloperoxidase in Hepatitis C Virus Infection and Associated Liver Cirrhosis

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Abstract: Myeloperoxidase (MPO) is an important enzyme that found in neutrophils and involved in reactive oxygen species (ROS) production. The aim of the current study was to clarify the potential role of MPO in oxidative stress and liver fibrosis associated with hepatitis C virus (HCV) infection. This study was conducted on 90 subjects, 10 normal controls and 80 patients having HCV infection classified into chronic hepatitis C without cirrhosis (CHC) (50 cases) and CHC with cirrhosis (LC) (30 cases). Myeloperoxidase was assessed in plasma by ELISA technique and in liver tissue by immunohistochemistry. Malondialdehye (MDA), as a marker of lipid peroxidation and oxidative stress was also measured in plasma by spectrophotometric assay. Results revealed significant increase of both plasma and hepatic tissue MPO in cirrhotic patients compared to either controls or CHC patients ($p<0.05$). Plasma and tissue MPO showed significant direct correlation with liver aminotransferases (ALT and AST), MDA and stage of hepatic fibrosis. Regression analysis revealed that both plasma and tissue MPO are independent determinant for MDA and stage of hepatic fibrosis. The results incriminate MPO in oxidative stress that causes tissue damage in chronic HCV patients and the subsequent development of hepatic cirrhosis.

Key Words: Myeloperoxidase, oxidative stress, chronic hepatitis C, cirrhosis, malondialdehye.

INTRODUCTION

Neutrophils are the most abundant circulating leukocytes. They constitute the first line of defense against non self substances that break through the body's physical barriers. Although neutrophils play a beneficial role in host immune defense, paradoxically they also have potential deleterious effects on normal host tissue [1]. Myeloperoxidase (MPO) is a dimeric enzyme composed of two heavy subunits (~ 60 Kd) and two light subunits (~15 Kd). It is found in primary (azurophil) granules of neutrocytes that involved in reactive oxygen species (ROS) production [2,3]. Reactive oxygen species are known to cause tissue damage and has attracted significant attention because it seems to be involved in a broad range of diseases [4]. More than 95% of plasma MPO is produced by activated neutrophils and the remaining is contributed to the less numerous monocytes in the circulation and tissue associated macrophages. Cytokines and growth factors induce myeloperoxidase gene expression in kupffer cells or cause monocytes to continue to express the enzyme as they differentiate into macrophages [5]. Because various acute and chronic inflammatory stimuli result in secretion of MPO into the blood [6], thus MPO can be used as an appropriate marker of neutrophil activation or degradation as in hypersplenism [7].

Hepatitis C virus (HCV) is a major cause of liver disease. It affects nearly 170 million people worldwide [8]. It is shown that oxidative stress may play a role in the pathogenesis of chronic hepatitis C (CHC) [9] and may regulate collagen synthesis, thus contribute to liver damage and the subsequent development of fibrosis [10,11]. Myeloperoxidase of neutrophils and monocyte-macrophages (Kupffer cells) catalyzes the reaction between chloride and hydrogen peroxide to generate hypochlorous acid (HOCl) and other ROS [5]. In response to these radicals many pro-inflammatory mediators are generated by the endothelium leading to expression of adhesion molecules that enroll leukocytes to become firmly adherent to the endothelium [12]. Activated and accumulated leukocytes produce a second burst of oxygen radicals, along with other neutrophil derived products, which cause further, and may be more severe damage [1]. Local release of oxidants together with cytokines, damage "bystander" hepatocytes that leads to activation of hepatic stellate cells [13]. Also, hepatic encephalopathy is exacerbated by increased production of reactive oxygen and nitrogen species which trigger multiple protein and RNA modification [14].

This study aimed to clarify the potential link of myeloperoxidase to oxidative stress and the associated tissue damage and/or fibrosis in chronic hepatitis C virus infection. Targeting this link may be beneficial not only in diagnosis but also in launching new therapeutic modalities. Nonetheless, without measuring parameters relevant to the status of antioxidant defenses and oxidative stress, it is not possible to determine whether the selection, dose, and duration of an

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antioxidant intervention achieves its intended biochemical or physiological endpoint or whether the enrolled subjects even present with oxidative stress.

SUBJECTS & METHODS

Study Design and Patients

Eighty (80) cases having HCV-related liver pathology were the subject of the current study. They were followed up by the clinicians at the Hepato-Gastroenterology Department, Theodor Bilharz Research Institute (TBRI), Giza-Egypt. Patients were 65 males and 15 females having a mean age of 45.6±6.4 (range 22-60 years). All had circulating anti-HCV antibodies or HCV-RNA viremia with no serologic evidence of co-infection with hepatitis B virus. All patients gave informed consent prior to participation in the study according to the institutions' human research ethics committee; and all procedures including liver biopsy were medically indicated for patient management.

The patients were subjected to thorough clinical examination and were assessed by: (1) laboratory investigations; (2) ultrasonography; and (3) blind liver biopsies which were done by Hepafix needle (Braun, Melsungen, Germany) for histopathologic and immunohistochemical studies.

The cases were divided into 50 patients having chronic hepatitis C virus infection without cirrhosis (CHC) and 30 patients having chronic hepatitis C with cirrhosis (LC). Children, schistosomal patients, chronic viral diseases other than HCV, nonalcoholic steatohepatitis, autoimmune hepatitis, biliary disorders, and malignancies were excluded from the study.

Ten control wedge liver biopsies were taken from age- and sex-matched individuals subjected to abdominal cholecystectomy after receiving their consent. They were 8 males and 2 females with a mean age of 42.8±6.6 years. Their liver function tests were within normal range. They had no serologic evidence of hepatitis B and/or C viruses and their liver biopsies were proved to be histopathologically free.

Laboratory Investigations

Blood samples were collected from all cases under complete aseptic conditions. One milliliter of blood was delivered into EDTA-containing tube to perform haemogram which include: White Blood Cell (WBCs) Count, Absolute Neutrophil Count (ANC), Red Blood Cell (RBCs) Count and Platelet (Plat) Count using automated cell counter (ACT differential, Beckman, France). Also, 1.8ml of blood was delivered into a tube containing 200ul Na citrate and centrifuged at 3000 rpm for 15 minutes. Plasma was separated and stored at -80∘C for the assay of both myeloperoxidase enzyme (MPO) and malondialdehye (MDA) levels. Plasma MPO was measured by enzyme linked immunosorbent assay (ELISA) technique (Bioxytech Oxis Research, USA) [15]; however, plasma MDA was determined by spectrophotometric assay (Oxis Research, USA) [16].

Two ml of blood was allowed to clot in a clean dry tube at room temperature, centrifuged at 2000 rpm for 10 minutes, and serum was separated for assessment of Liver function tests including: Alanine aminotransferse (ALT), aspartate aminotranferse (AST) and albumin (Alb) using auto-analyzer (Hitachi 736, Japan); and Viral hepatitis markers including: Hepatitis B surface antigen and hepatitis B core antibody using enzyme immunoassay kits (Abbott Laboratories; North Chicago, Illinois). Circulating anti-HCV antibodies were detected using Murex enzyme immunoassay kit (Murex anti-HCV, Version V; Murex Diagnostics; Dartford, England).

Chronic hepatitis C (CHC) was confirmed by HCV infection persisting for longer than 6 months and proved by reverse-transcriptase polymerase chain reaction (HCV-RNA positive) as well as increased ALT values.

Histopathological Studies

Serial sections (5-μm thick) from formalin-fixed, paraffin-embedded core liver biopsies were stained with hematoxylin & eosin for routine histopathologic examination. Masson trichrome stain was used for proper demonstration of fibrous tissue deposition. The stage of hepatic fibrosis was evaluated in a scale of (0-6) [17].

Immunohistochemical Detection of Hepatic Myeloperxidase (MPO)

The 5-μm thick sections from formalin-fixed, paraffin-embedded blocks were cut on microscopic slides coated with 3-amino propyl triethoxysilane (Sigma Chemicals; St. Louis, Missouri) for proper fixation of tissue sections on the slides and to minimize staining artifacts. Following de-paraffinization in xylene and rehydration, endogenous peroxidase activity was quenched by incubation in 0.3% v/v H_2O_2 in methanol for 20 minutes at room temperature. Antigen retrieval was performed by microwaving in 10 mmol/L citrate buffer solution (pH 6.0). Non-specific antibody binding was prevented by application of blocking serum for 30 minutes. Sections were incubated overnight with the ready to use N-series primary antibody (polyclonal rabbit anti-human myeloperoxidase antibody, Code N1578, Dako Cytomation). After thorough washings in phosphate buffer saline, the bound antibody was detected with biotinylated anti-rabbit IgG, followed by streptavidine peroxidase conjugation. Brown color was developed using DAB substrate. Sections were counterstained with Mayer's hematoxylin before mounting. Positive and negative controls were stained appropriately in the same setting.

The immunohistochemical expression of MPO in liver biopsies was graded as negative (-), mild (+=<15 cells/40 magnification), moderate (++=15-30 cells/40 magnification) or marked (+++=>30 cells/40 magnification) per section [4].

Statistical Analysis

Statistical Package for Social Sciences (SPSS) for Windows, version 11, was used for statistical analysis. Means and standard deviation of studied parameters were compared in different groups using one-way ANOVA. Comparison between percent (%) of positive cases was done using Chi-square test. Pearson correlation coefficient "r" was used to measure the relationship between two variables. Stepwise multiple linear regression analysis was employed to evaluate any association between MPO and oxidative stress or tissue fibrosis. For all tests, a "p" value less than 0.05 was considered statistically significant.

Results

Patients of the study were mainly males of middle age presented with pallor and/or jaundice, fatigue, myalgia and right hypochondrial pain. However, in cirrhotic patients; palmer erythema, spider naevi and lower limb edema were also evident. Thirteen out of fifty CHC patients (26%) and fourteen out of thirty LC patients (46.7%) had splenomegaly (Table **1**).

ALT and AST were significantly elevated in both CHC and LC patients compared to controls ($p<0.05$). Also, a significant rise was detected in LC compared to CHC ($p<0.05$). However, serum albumin level was significantly reduced in LC compared to both controls and CHC patients ($p<0.05$) with no significant difference between CHC and controls (Table **2**).

Plasma MPO level was significantly elevated in LC compared to both controls and CHC patients ($p<0.05$) with no significant difference between CHC and controls (Table, **2**). In CHC, plasma MPO showed no significant difference with either the presence or absence of splenomegaly. But in LC, plasma MPO was significantly increased in patients with splenomegaly compared to those without splenomegaly ($p<0.05$). Correlation analysis revealed that plasma MPO had a significant reverse correlation with ANC, RBCs, and platelet counts (r=-0.605, -0.564, and -0.567 respectively).

On the other hand, both plasma MDA level and tissue MPO expression were significantly elevated in CHC and LC patients compared to controls ($p<0.05$) with a statistical significant difference between CHC and LC ($p<0.05$) (Table, **2**).

Table 1. Demographic, Clinical and Ultrasonographic Findings of all Studied Cases

Variables	Control Cases (*n* = 10)	CHC (*n* = 50)	LC (*n* = 30)
Age **(Mean±SD)**	42.8±6.6	44.4±6.1	46.8±6.7
Gender **(M:F)**	4:1	4:1	4:1
Clinical findings: *n* **(%)**			
- Jaundice	0 (0)	13 (26%)	12 (40%)
- Hepatomegaly	0 (0)	16 (32%)	13 (43.3%)
- Splenomegaly	0 (0)	13 (26%)	14 (46.7%)
Ultrasound findings: *n* **(%)**			
- Hepatomegaly	0 (0)	21 (42%)	12(40%)
- Liver echogenicity			
Normal	10 (100)	26 (52%)	10(33.3%)
Hyperechoic	0 (0)	24 (48%)	20(66.6%)
- Splenomegaly	0 (0)	13 (26%)	14 (46.7%)

CHC= Chronic hepatitis C. LC= Liver cirrhosis.
n= Number SD=Standard deviation M:F= Male: Female %=percent.

Table 2. Laboratory Results of All Studied Cases

	Controls (*n*=10) (Mean±SD)	CHC (*n*=50) (Mean±SD)	LC (*n*=30) (Mean±SD)
Serum ALT (U/L)	19.20±2.73	39.34±1.01[a]	52.63±1.27[ab]
Serum AST (U/L)	19.90±2.8	38.06±0.97 [a]	51.13±1.23[ab]
Serum Alb (g/dL)	4.29±0.12	3.93±0.08	2.9±0.09[ab]
Plasma MPO (ng/mL)	200±9.03	199.94±4.34	419.53±22.68[ab]
Plasma MDA (nmol/mL)	3.75±0.45	10.69±0.32[a]	14.07±0.22[ab]
Tissue MPO	9.30±2.63	17.32±6.96 [a]	33.13±13.30 [ab]

a: Significant difference compared to control cases ($p<0.05$).
b: Significant difference compared to CHC ($p<0.05$).
Normal range for Alanine aminotransferse (ALT) and aspartate aminotranferse (AST) was: 5-37 U/L and for albumin (Alb) was: 3.5-5 g/dL.
SD=Standard deviation. *n*= Number.

Immunostaining revealed myeloperoxidase expression in kupffer cells of all studied liver biopsies. Although the number of positively stained cells varied among the cases, it was mild (+) in all normal controls with almost negligible immunoreactivity in hepatocytes (Table **3**). The number of cells expressing myeloperoxidase was increased in CHC patients (Tables **2** & **3**) with statistical significant difference compared to controls (p<0.05). The immunoreactive kupffer cells in CHC were slender and conformed to the contour of the sinusoidal lumen (Fig. **1**); however, others had enlarged nuclei that protruded into the lumen (Fig. **2**). Morphologically these cells were the quiescent and activated kupffer cells, respectively. Polymorphonuclear leukocytes within sinusoidal lumina were also reactive with the antibody. The pattern of immunoreactivity was different in cirrhotic livers. Numerous spindle-shaped cells of the fibrous septa and the adjacent hepatocytes were positive for myeloperoxidase (Figs. **3** & **4**). Spindle cell immunoreactivity was accentuated in areas of bile duct proliferation. With marked expression, a statistical significant increase of hepatic MPO positivity was observed in LC compared to CHC cases (p<0.05) (Table **4**).

It was found that myeloperoxidase immunoexpression was increased with progression of hepatic fibrosis where cases of established cirrhosis (stages 5 and 6), showed marked staining reaction compared to lower stages with significant statistical difference (p<0.05) (Table **4**).

There was direct correlation between plasma and tissue MPO (r=0.450). Both plasma and tissue MPO showed a significant direct correlation with liver function tests namely serum ALT (r=0.772 and 0.416 respectively) & serum AST (r=0.787 and 0.416 respectively); and a significant reverse correlation with serum albumin (r=-0.658 and -0.374, respectively). Also both plasma and tissue MPO showed a significant direct correlation with plasma MDA (r=0.727 and 0.467 respectively) as well as the stage of hepatic fibrosis (r=0.720 and 0.777 respectively) (Table **5**).

Table 3.   Immunohistochemical Hepatic Expression of Myeloperoxidase in all Studied Cases

Histopathologic Diagnosis	No. of Cases (*n*=90)	Myeloperoxidase Tissue Expression		
		Mild (+) *n* (%)	Moderate (++) *n* (%)	Marked (+++) *n* (%)
Controls	10	10 (100)	0 (0)	0 (0)
CHC	50	30 (60)	18 (36)	2 (4)
LC	30	4 (13.3)	10 (33.3)	16 (53.4)*

* Significant difference compared to CHC cases (p<0.05).
n= Number %= percent CHC= Chronic hepatitis C. LC= Liver cirrhosis.

Table 4.   Immunohistochemical Hepatic Expression of Myeloperoxidase According to the Stage of Hepatic Fibrosis in Diseased Patients

Stage of Fibrosis	No. of Cases (*n*=80)	Myeloperoxidase Tissue Expression		
		Mild (+) *n* (%)	Moderate (++) *n* (%)	Marked (+++) *n* (%)
1-2	25	17 (68)	8 (32)	0
3-4	25	13 (52)	10 (40)	2 (8)
5-6	30	4 (13.3)	10 (33.3)	16 (53.4)*

* Significant difference compared to lower stages of fibrosis (p<0.05).
*n*= Number %= percent.

Table 5.   Correlation Analysis of Plasma and Tissue Myeloperoxidase with Liver Function Tests and MDA

|	Tissue MPO	Serum ALT	Serum AST	Serum Alb	Plasma MDA	Stage of Fibrosis
Plasma MPO	r=0.450*	r=0.772*	r=0.787*	r=-0.658*	r=0.727*	r=0.720*
Tissue MPO	---------	r=0.416*	r=0.416*	r=-0.374*	r=0.467*	r=0.777*

*Significant difference (p<0.05)
Myeloperoxidase (MPO), alanine aminotransferse (ALT), aspartate aminotranferse (AST), albumin (Alb), malondialdehye (MDA).
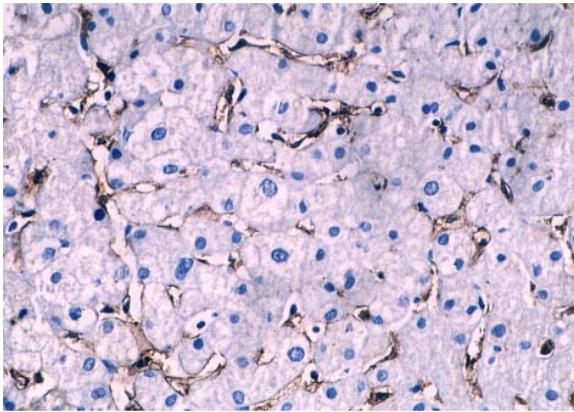
Fig. (1). Immunoreaction for MPO in the slender quiescent kupffer cells of chronic hepatitis C (Immunostain X40).
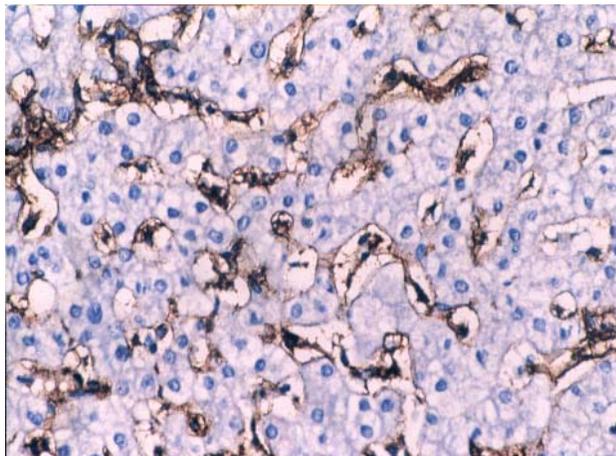
Fig. (2). MPO expression in the activated kupffer cells with enlarged nuclei protruding into the lumen in chronic hepatitis C (Immunostain X40).
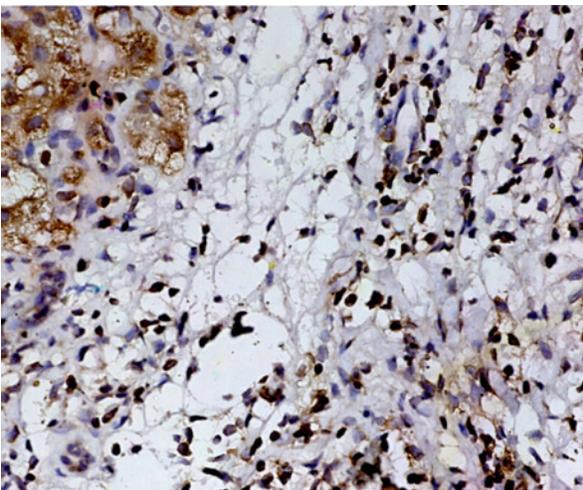

Fig. (3). Marked MPO expression in the numerous spindle-shaped cells of the fibrous septa and in the hepatocytes of the cirrhotic nodules (Immunostain X20).

DISCUSSION

Small amounts of reactive oxygen species (ROS) are constantly produced in aerobic metabolism that have important role in normal cell physiology. However, in pathophysi-ologic conditions with increased levels of ROS, these molecules become relevant factors in the initiation and amplification of deleterious processes observed in inflammation and degenerative diseases [18,19]. It was found that hepatitis C virus infection is characterized by remarkable levels of oxidative stress [20,9]. There is a relationship between HCV load and antioxidant status. The association may reflect consumption of antioxidants due to HCV, although effects of low antioxidant status on viral replication cannot be excluded [21]. Osterreicher *et al.* and Galli *et al.* [13,22] reported that oxidative stress plays a central role in the progression of hepatitis to cirrhosis. Also, it plays an important role in the pathogenesis of liver failure [23,24]. Furthermore, oxidant production associated with viral hepatitis leads to mutations in DNA and RNA which subsequently leads to hepatocellular carcinoma [25,26].
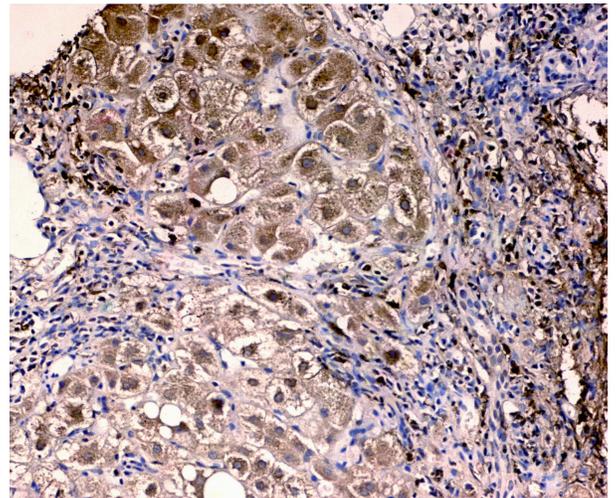
Fig. (4). Higher power view of LC case showing expression in the spindle-shaped cells of the fibrous septa and adjacent hepatocytes (Immunostain X40).

This study supports the role of MPO in the pathogenesis of hepatitis C virus infection and its sequel of liver cirrhosis. Our results demonstrated that plasma MPO was unequivocally increased in cirrhotic patients especially with splenomegaly compared to normal controls or chronic hepatitis C cases. There are two possible explanations for these results either due to: increased neutrocytic destruction in the associated hypersplenic conditions with the release of large amount of neutrocytic MPO; or due to decreased hepatic clearance of MPO because of liver dysfunction and/or portosplenic shunting. Neutrocytopenia is one of the frequent complications of hyperslenism in patients with cirrhosis that resulted from increased degradation of neutrocytes and/or their excessive pooling within the spleen [7]. Correlation analysis of our results revealed that plasma MPO has a significant reverse correlation with neutrophil, red cell and platelet counts; accordingly, it can be used as a marker of hypersplenism in these patients.

On the other hand, the expression of MPO in liver tissue revealed mild reactivity in control cases. Meanwhile, moderate to marked expression was observed in CHC and LC patients with a significant increased expression in cirrhotic livers. The significant direct correlation between tissue MPO and liver aminotransferases (ALT and AST) together with

the reverse correlation with serum albumin also support the role of MPO in hepatitis C virus infection.

Malondialdehyde (MDA) is the most important end product of free radical reactions. Its plasma level is widely used as a marker of lipid peroxidation [27-29]. In the current study, plasma MDA was increased in CHC and LC patients with a significant rise in cirrhotic patients compared to normal controls or chronic hepatitis cases. The significant direct correlation between both plasma and tissue MPO with plasma MDA, emphasizes the impact of MPO in oxidative stress and tissue damage associated with chronic hepatitis C virus infection.

Increased concentrations of ROS may lead to tissue damage by several mechanisms that cause direct modification of cellular components, cell structure and function. Oxidation of lipids generates lipid radicals that can derange cell and basement membranes because their orderly architecture and integration depend on non-oxidized lipids [30,31]. However, oxidative modification of protein residues can promote the loss of scaffolding property of structural proteins, inactivate enzymes and alter the degradation and clearance of these molecules [18] and oxidation of purines, pyrimidines and the attached ribose moiety can alter gene expression [32]. Increased ROS may act as trans and intercellular signals that activate or inactivate redox-sensitive protein kinases or phosphatases resulting in altered phosphorylation of receptors and transcription factors. This can lead to changes in the expression of cytokines, adhesion molecules and proteins involved in proliferation and apoptosis, thereby affecting inflammation, cell to cell contact and cell death [33-35].

Myeloperoxidase may stimulate production of extracellular matrix by kupffer cells. Alteration of extracellular matrix has been linked to the activation of hepatic stellate cells, which is a critical step in the development of hepatic fibrosis. This was proved in this study by increased MPO tissue expression in kupffer cells as the fibrosis progress. It was also found that, hepatic fibrosis was associated with increased number of myeloperoxidase-expressing macrophages in connective tissue septa where 53.4% of cases with established cirrhosis (stages 5 and 6), showed significantly marked staining expression compared to lower stages. This explains the role of free radicals produced by MPO in the pathogenesis of liver cirrhosis as previously recorded [36-38]. Stepwise multiple regression analysis of the results of the current study revealed that both plasma and tissue MPO were independent determinants for MDA and stage of hepatic fibrosis in chronic HCV infection. Thus reactive species generated by myeloperoxidase may in turn peroxidize lipids, thereby enhancing collagen production by activated stellate cells. These observations point to a novel oxidative pathway by which kupffer cells could participate in liver injury.

CONCLUSION

It could be concluded that high level of plasma MPO, associated with increased hepatic tissue MPO immunoreactivity have an important impact in oxidative stress in HCV patients and play a critical role in the development of hepatic cirrhosis.

Biochemical defenses against the products of myeloperxidase have therapeutic implications that differ from those that prevent damage by other reactive species.

However, further studies are recommended to evaluate the benefits of antioxidants targeting MPO in the pathophysiological changes associated with chronic HCV infection.

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