HEPATOLOGY

Iron overload in patients with chronic hepatitis C virus infection: Clinical and histological study

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Abstract

Background: Recently it has been found that iron is an important element in the natural history of hepatitis C. Serum markers of iron stores are frequently increased in chronic hepatitis C virus (HCV)-infected carriers but the real impact of the hepatic iron overload is poorly understood. The purpose of the present paper was to determine the prevalence of iron overload and to study the relationship between hepatic iron concentration (HIC) and clinical, biochemical and histological characteristics in chronic HCV-infected carriers.

Methods: Patients presenting with anti-HCV and HCV-RNA were included. Hepatic iron concentration was determined in liver tissue by atomic absorption spectrophotometry. The association between HIC and age, gender, risk factor of transmission, duration of infection, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, iron and serum ferritin, transferrin saturation, HCV-RNA level, grading of inflammatory activity, staging of fibrosis, hepatic steatosis, and stainable iron was analyzed. Statistical analysis included the Mann–Whitney test and a multiple linear regression model.

Results: Ninety-six patients (58% male) with a mean age of 44 ± 10 years were studied. Serum iron, ferritin and transferrin saturation were elevated in 28%, 27% and 12.5% of patients, respectively. Stainable iron was detected in few patients (15.6%). Higher grades of stainable iron (2 and 3) were observed in only 7%. The HIC (>30 mmol/g dry weight) was elevated in five patients (5%). Neither grading nor staging were related to HIC. Higher HIC were observed in male patients (P < 0.001), in patients with elevated serum ferritin (P = 0.001) and in patients with stainable iron (grades 2 and 3; P = 0.001). Multiple linear regression analysis showed that only stainable iron was independently correlated with HIC (P = 0.003).

Conclusions: Iron overload in chronically HCV-infected patients was uncommon and hepatic iron content seemed not to be related to the liver damage process. In the eventuality of iron overload, histochernical liver iron is a useful marker to estimate HIC.

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INTRODUCTION

Most of the hepatitis C virus (HCV)-infected patients develop a chronic slowly progressive liver disease that may result in cirrhosis and hepatocarcinoma. Several factors have been proposed to explain this unfavorable evolution such as male gender, age at infection and alcohol abuse. Recently, the role of iron has been pointed out as an important element in the natural history of hepatitis C. In fact, serum iron stores are frequently increased in chronic HCV-infected carriers but little is known about the significance of these abnormalities.

The iron excess in hepatitis C may be due to hereditary hemochromatosis, hematologic diseases, multiple transfusions, porphyria cutanea tarda and chronic alcohol abuse. However, if these factors are absent the mechanisms involved in iron overload remain unclear.
Over the last few years, there has been much interest in the study of iron in hepatitis C because some studies have shown that iron accumulation in chronic HCV carriers is related to a poor response to interferon therapy.\textsuperscript{6,7} In addition, others have suggested that hepatic iron overload in hepatitis C may worsen liver damage by still unrecognized mechanisms.\textsuperscript{10,11}

Iron overload is often assessed using indirect parameters of iron stores such as serum iron, serum ferritin and transferrin saturation, which are frequently increased in chronic viral hepatitis,\textsuperscript{2} as well as by the histological determination of stainable iron in liver samples.\textsuperscript{12} Nevertheless, these measures frequently do not determine hepatic iron content accurately, with possible misconceptions about the diagnosis of iron overload. Therefore, in order to study iron overload in chronic HCV carriers we assessed hepatic iron concentration (HIC) and verified the influence of HIC on clinical, biochemical and histological profile in these patients.

**METHODS**

**Patients**

The study included consecutive patients with chronic HCV infection who tested positive for anti-HCV and HCV-RNA seen at the Hepatitis Division of the Universidade Federal de São Paulo between May 2000 and September 2001. Patients were evaluated in terms of clinical characteristics, biochemical data, HCV viral load, histological features and iron status parameters. Exclusion criteria were as follows: contraindications to liver biopsy, chronic alcohol consumption >20 g/day, HBV/HIV coinfection, immunosuppression, previous antiviral therapy, history of multiple blood transfusion (>6 U), chronic renal disease, unsuitable sample liver biopsy, history of hemolytic anemia, and therapy with iron.

**Clinical characteristics**

Patients were evaluated according to their mean age, gender, route of transmission and mean duration of infection. The mode of transmission was considered parenteral in those patients presenting with a history of blood product transfusion and/or intravenous drug use (IVDU). The duration of infection was estimated based upon the year of the first transfusion or the first year of intravenous drug use.

**Biochemical data**

Alanine aminotransferase (ALT, U/L) and aspartate aminotransferase (AST, U/L) were determined in serum samples obtained 1 day before the liver biopsy. For comparisons we considered values of ALT and AST as an index based upon the upper limit of the normal (ULN) range, classifying patients as normal (≤1 × ULN) or as elevated (>1 × ULN).

**Hepatitis C virus assays**

Anti-HCV was detected in serum samples by a third-generation ELISA (Abbott Laboratories, IL, USA). Qualitative HCV-RNA polymerase chain reaction was performed by the Amplicor HCV Test (Roche Diagnostic System, USA). The HCV-RNA viral load was obtained in 63 patients using the Amplicor HCV Monitor Test, version 2.0 (Roche Diagnostic System). The lower limit of detection was 600 IU/mL. For comparison patients were classified according to viral load below or above 800,000 IU/mL.

**Histological examination**

A liver biopsy was obtained from all patients using a Tru-cut needle. The samples were cut into two parts. A portion of the tissue fragment was immediately frozen at −20°C for subsequent determination of iron content, and the other was used for histopathological examination. The latter specimens were formalin-fixed and paraffin-embedded. Slides were stained with hematoxylin–eosin, Masson’s trichrome, Perls’ Prussian blue and Gomori reticulin stain. One pathologist who was unaware of the patients’ clinical and laboratory data performed the histopathological examination. The histological study included the analysis of the grade of portal/perportal necro-inflammatory activity and the stage of fibrosis, which were assessed using a semiquantitative scoring system according to Desmet \textit{et al.}\textsuperscript{13} For comparison, grading of inflammatory activity and staging of fibrosis were grouped into grade 0–2 versus grade 3–4. Hepatic steatosis, defined as fat accumulation within hepatocytes, was also determined in all patients.

**Iron status parameters**

\textit{Analysis of iron status by serum tests}

Serum iron status was assessed in blood samples obtained from each patient after an overnight fast 1 day before the liver biopsy to measure serum iron, serum ferritin and transferrin saturation. Serum iron was measured by the Ferene-S method (normal for female subjects: 37–145 μg/dL; normal for male subjects: 59–158 μg/dL). Serum ferritin was analyzed by an immunometric assay (normal for women aged 18–45 years, 6–115 ng/mL; normal for women aged >45 years, 5–200 ng/mL; normal for men aged 18–45 years, 22–340 ng/mL; normal for men aged >45 years, 22–415 ng/mL). For comparison, we considered values of serum iron and ferritin as an index based upon the ULN range, classifying patients as normal (≤1 × ULN) or as elevated (>1 × ULN). Transferrin was determined by immunoturbidimetric method using Cobas Mira Instruments (USA; normal: 200–400 mg/dL). Transferrin saturation (TS) was calculated as follows: (serum iron/serum transferrin) × 71.2 and values were expressed as percentage (%).\textsuperscript{14} The TS was considered elevated at values >45%.
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Assessment of stainable liver iron content

A grading system was applied to liver samples stained with Perls' Prussian blue to assess liver iron content. Stainable iron was scored on a scale of 0–4 as follows: grade zero, no detectable iron; grade one, granules of iron visible only at 400× magnification; grade 2, discrete iron granules visible at 100×; grade 3, iron visible at 25×; and grade 4, masses of iron visible to the naked eye or at 10× magnification. Comparisons were made between patients with stainable liver iron grade ≤1 versus stainable liver iron grade >1.

Assessment of hepatic iron concentration

To measure HIC, fresh liver specimens placed in a clean tube were dried to constant weight in a vacuum oven at 52°C. The dried samples were then weighed and placed in a tube containing 1 mL concentrated nitric acid and 1 mL ion-free water. Next, the samples were digested in a microwave digestion system (MDS 2000, USA). A blank and samples of bovine liver used as a reference material (1577b) were treated similarly. The resulting solutions were transferred to volumetric flasks and made up with ion-free water. All digested samples were diluted 1:8 in 0.2% nitric acid and a 10-μL volume was injected into the furnace for measurement. Iron concentration was determined by graphite furnace atomic absorption spectroscopy using a Varian Perkin-Elmer SIMAA-6000 spectrometer (USA). The results are expressed as μmol/g dry weight. The HIC > 30 μmol/g dry weight was considered to be elevated. The hepatic iron index was also calculated by dividing HIC by patient age, with the abnormal range being >1.9. For comparisons, patients were analyzed according to median HIC values.

Statistical analysis

Results are expressed as mean ± SD and median with their ranges. First, the HIC determined in all patients was compared to mean age, gender, risk factor of transmission, duration of infection, serum ALT and AST levels, serum iron, serum ferritin, transferrin saturation, HCV viral load, grading of inflammatory activity, staging of fibrosis, hepatic steatosis and stainable liver iron, using the Mann–Whitney U-test. Then, a multiple linear regression model was applied to look for independent factors correlated with HIC. Differences were considered significant for P < 0.05. SPSS 10.0 (SPSS, Chicago, IL, USA) was used for data analysis.

Informed consent

The study was carried out in accordance with the Helsinki Declaration. All the patients selected for the study, after being briefed about the research, gave their informed consent.

RESULTS

A total of 96 patients with chronic HCV infection were enrolled in the study.

Clinical, biochemical and histological characteristics

The clinical, biochemical, HCV load viral and histological patient characteristics are shown in Table 1.

Iron status parameters

Serum iron, serum ferritin, transferrin saturation, HIC and HII are presented in Table 2. The dry weight of liver samples ranged from 0.4 mg to 9 mg, with a mean of 2.6 ± 1.7 mg and a median of 2 mg.

Stainable liver iron was present in only 15 of 96 patients (15%; Table 1). Iron stain grade 4 was not found in any patient.

Comparisons between HIC and patient clinical and biochemical data and HCV viral load

Comparisons between HIC and clinical aspects of the patients showed no significant difference with respect to

Table 1 Clinical, biochemical, virological and histological characteristics of patients with chronic HCV infection

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n = 96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at biopsy (years) †</td>
<td>44 ± 10</td>
</tr>
<tr>
<td>Gender: male/female</td>
<td>56/40</td>
</tr>
<tr>
<td>Parenteral risk for HCV infection</td>
<td></td>
</tr>
<tr>
<td>Transfusion</td>
<td>39</td>
</tr>
<tr>
<td>IVDU</td>
<td>12</td>
</tr>
<tr>
<td>Others</td>
<td>45</td>
</tr>
<tr>
<td>Duration of infection (years) ‡‡</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>HCV-RNA levels†§</td>
<td></td>
</tr>
<tr>
<td>≤800,000 IU/mL</td>
<td>45</td>
</tr>
<tr>
<td>&gt;800,000 IU/mL</td>
<td>18</td>
</tr>
<tr>
<td>AST (mean × ULN)</td>
<td>1.8 ± 1.9</td>
</tr>
<tr>
<td>ALT (mean × ULN)</td>
<td>2.5 ± 2.4</td>
</tr>
<tr>
<td>Histological findings</td>
<td></td>
</tr>
<tr>
<td>Without HC</td>
<td>8</td>
</tr>
<tr>
<td>HC with minimal and mild activity</td>
<td>55</td>
</tr>
<tr>
<td>HC with moderate and severe activity</td>
<td>21</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>12</td>
</tr>
<tr>
<td>Hepatic steatosis§</td>
<td>62</td>
</tr>
<tr>
<td>Stainable iron</td>
<td></td>
</tr>
<tr>
<td>grade zero</td>
<td>81</td>
</tr>
<tr>
<td>grade 1</td>
<td>8</td>
</tr>
<tr>
<td>grade 2</td>
<td>5</td>
</tr>
<tr>
<td>grade 3</td>
<td>2</td>
</tr>
</tbody>
</table>

IVDU, intravenous drug use; AST, aspartate aminotransferase; ALT alanine aminotransferase; HC, chronic hepatitis; ULN, upper limit of normal.

†Levels expressed as mean ± SD; ‡HCV viral load determined in 63 patients; §hepatic steatosis was found in 62 among 96 patients.
age ($P = 0.5$), risk of transmission ($P = 0.8$) or duration of infection ($P = 0.6$). In contrast, median HIC values were higher in men than in women ($P < 0.001$).

No relationship was found between HIC and AST ($P = 0.4$) or between HIC and ALT ($P = 0.2$). Comparisons between HIC and HCV viral load showed that the median HIC values were higher in patients with elevated serum HCV-RNA levels, although the difference was not significant ($P = 0.06$). Patients with high viral load had a median of HIC of 12 μmol/g dry weight versus patients with low viral load with a median of HIC of 5.2 μmol/g dry weight.

**Comparisons between HIC and histological features**

No significant relationship was found between HIC and histology grading of inflammatory activity ($P = 0.2$) or with staging of fibrosis ($P = 0.2$). In addition, HIC values were not different between patients with or without liver steatosis ($P = 0.8$). The HIC values according to grade of inflammatory activity and staging of fibrosis are shown in Fig. 1.

**Comparisons between HIC and iron status parameters**

The HIC and serum iron did not differ significantly ($P = 0.07$). Similarly, patients with transferrin saturation $>45\%$ tended to have higher HIC values although this difference was not significant ($P = 0.08$). In contrast, HIC was significantly higher in patients with elevated serum ferritin ($P = 0.001$).

When HIC values were compared to stainable liver iron, we could see that patients with grade 2 and 3 stainable iron had higher HIC ($P = 0.001$). The distribution of HIC values according to liver stainable iron is shown in Fig. 1.

**Final model of multivariate analysis**

Gender, serum iron, serum ferritin, TS and stainable liver iron were the variables included in the multiple linear regression model. As a final result, we observed that liver grade 2 and 3 stainable iron was the only variable independently associated with HIC values ($r = 0.304; P = 0.003$).
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Table 3 Results of comparisons between HIC and characteristics of 96 chronic HCV-infected patients

<table>
<thead>
<tr>
<th>Preliminary results</th>
<th>HIC (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HCV-RNA viral load†</td>
<td>0.06</td>
</tr>
<tr>
<td>Serum iron</td>
<td>0.07</td>
</tr>
<tr>
<td>Serum ferritin</td>
<td>0.001</td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>0.08</td>
</tr>
<tr>
<td>Stainable iron</td>
<td>0.001</td>
</tr>
<tr>
<td>Final result</td>
<td></td>
</tr>
<tr>
<td>Stainable iron (grade 2 and 3)‡</td>
<td>0.003</td>
</tr>
</tbody>
</table>

†HCV viral load determined in 63 patients. ‡The only variable independently associated with HIC by multivariate linear regression;

The first and final results of the comparative study between HIC and characteristics of patients with chronic HCV infection are given in Table 3.

DISCUSSION

The occurrence of a significant number of patients with elevated serum iron (28%), serum ferritin (27%) and transferrin saturation (12.5%) in the present study confirms the observations reported by other authors that these changes are commonly seen in chronic HCV-infected patients.3,15 Although the present findings may indicate the presence of iron overload among the patients studied, the HIC in liver tissue samples considered as the gold standard diagnosis revealed that only 5% of patients had iron overload, defined as HIC ≥ 30 μmol/g dry weight.

Different rates of elevated HIC, ranging from 3% to 30%, have been previously reported.15-18 The small number of cases with elevated HIC values in the present study may be explained in part by the exclusion of factors that could favor the development of hepatic iron accumulation such as multiple transfusions, hemolytic anemia, alcohol abuse and iron therapy.

Comparisons between HIC and clinical characteristics of the patients showed that HIC values were significantly higher among men, an expected finding because men are not exposed to iron loss caused by menstruation.

Hepatic iron is thought to increase with time, especially in hereditary hemochromatosis. In the present study, however, patient age or duration of infection were not found to be correlated with higher HIC values. Therefore, in chronic HCV infection iron accumulation does not appear to be an event that intensifies with time.

There was no significant difference between HIC and route of transmission, as also reported by other authors.16,19

Several mechanisms have been proposed to explain the relation between HCV infection and hepatic iron overload. Some have suggested that hepatic iron accumulation results from release of iron from damaged liver cells.2 However, in the present study HIC was not different when compared to aminotransferase or to histological grading of inflammatory activity. Moreover, it has been suggested that iron may promote HCV replication.20,21 Such a finding was not demonstrated in the present study because no relationship was found between HCV-RNA level and HIC value: only a tendency was detected. Perhaps the association could be demonstrated if more patients were analyzed for viral load.

It is known that iron overload has a toxic effect on tissues and cells. In the common form of hereditary hemochromatosis hepatic iron overload results in several complications including cirrhosis, which develops at a HIC > 16 000 μg/g dry weight.22 In hepatitis C a synergistic effect of iron in liver tissue and the viral infection may result in advanced hepatic disease, even in the presence of a lower hepatic content.23,24 In the current study such a phenomenon was not detected, given that no relationship was seen between HIC values and stage of liver fibrosis.

In addition to histological features, no relationship was found between HIC values and patients with or without hepatic steatosis. Some studies have suggested that hepatic steatosis in hepatitis C results from oxyradical species and lipid peroxidation as an effect of excess iron within tissues.25 Our result may suggest another pathway involved in the development of this lesion.

In the current study, serum iron and transferrin saturation were not good markers of hepatic iron content. An association between elevated serum ferritin and higher HIC values was detected, but in the final model of analysis serum ferritin did not remain as an independent variable associated with HIC. This result confirms the assumption that ferritin is an inadequate marker of hepatic iron content because it increases in acute and chronic inflammatory processes with or without liver damage.26,27

The HIC was higher in patients with a more intense degree of stainable iron (grade 2 and 3), but after multivariate linear regression the finding of more intense degrees of stainable iron was the only variable that remained independently associated with a higher HIC value. This result may suggest that the evaluation of hepatic iron content in patients with chronic HCV infection may be estimated by the analysis of stainable iron in liver samples, especially with more intense degrees of stainable iron.

In summary, the current study showed that hepatic iron overload was an uncommon event in patients with chronic HCV infection. Moreover, serum iron, serum ferritin and transferrin saturation were not good as indirect markers of hepatic iron content. In contrast, the analysis of stainable liver iron seemed to be more adequate to assess iron in liver tissue. There was no evidence in the present study for the effect of hepatic iron content on the progression of hepatic disease toward cirrhosis due to HCV infection. The patients studied may have developed liver damage as a result of the natural history of hepatitis C independently of iron content within liver tissue.
REFERENCES