The influence of hepatitis C and iron replacement therapy on plasma pentosidine levels in haemodialysis patients

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Abstract

Background. Chronic liver disease and intravenous (i.v.) iron therapy can enhance oxidative stress. The aim of this study was to assess the influence of hepatitis C virus (HCV) and i.v. iron administration on oxidative stress in chronic haemodialysis (HD) patients.

Methods. A total of 115 HD patients (47% males, age 47±13 years) were placed in two groups according to the presence (HCV+) or absence (HCV/C0) of serum antibodies against HCV. Plasma pentosidine, high sensitivity C-reactive protein (hsCRP), interleukin-6 (IL-6) and alanine aminotransferase (ALT) levels were measured. The patients were also analysed according to the tertiles of serum levels of ferritin: group 1 (ferritin < 380 ng/ml), group 2 (ferritin 380–750 ng/ml) and group 3 (ferritin > 750 ng/ml). The cumulative iron dose was recorded during 6 months prior to the study.

Results. HCV+ patients had significantly higher levels of plasma pentosidine and ALT than HCV/C0 patients. Age, gender, serum albumin, IL-6 and hsCRP did not differ according to HCV serology. The levels of pentosidine were related to the ferritin levels and were significantly higher in group 3 compared with group 1. Moreover, the cumulative dose of iron was significantly higher in group 3 than in group 1. Plasma pentosidine showed a positive correlation with age, HCV and ferritin. In a stepwise backward multiple regression model, age and HCV were independent predictors of pentosidine levels.

Conclusion. HCV in HD patients is associated with increased pentosidine levels, possibly reflecting increased oxidative stress. The association between pentosidine and ferritin levels may suggest an impact of i.v. iron therapy.

Keywords: ferritin; haemodialysis; hepatitis C; oxidative stress; pentosidine

Introduction

In uraemia, the circulating levels of advanced glycation end-products (AGEs) are elevated and may promote atherosclerosis [1]. AGEs constitute a heterogeneous class of structures, such as pentosidine, Nε-carboxymethyl-lysine and imidazole, which are formed by non-enzymatic glycation and oxidation reactions between carbohydrate-derived carbonyl compounds, and protein and amino groups (Maillard reaction). The accumulation of AGEs in chronic kidney disease (CKD) patients seems to be independent of serum glucose, but is associated with inflammation, oxidative stress, malnutrition and low residual renal function (RRF) [2,3].

Hepatitis C virus (HCV), which infects 170 million people worldwide, is not an unusual condition in Brazilian haemodialysis (HD) patients. The prevalence of HCV antibodies is high in Brazilian HD centres (range 20–50%) [4]. Increased oxidative stress is a common feature of chronic liver disease [5] and, in patients with HCV infection, increased levels of markers of lipid peroxidation have been demonstrated in the liver, serum and leukocytes [6].

Anaemia in patients with CKD is usually treated by administration of subcutaneous erythropoietin (EPO)
and intravenous (i.v.) iron therapy. Recently, it has been suggested that administration of large doses of parenteral iron may be associated with increased morbidity and mortality in HD patients [7]. Moreover, iron increases the formation of reactive oxygen species, leading to lipid peroxidation. The aim of this study was to investigate the influence of serological HCV status and iron replacement therapy on pentosidine levels in a cohort of HD patients.

Subjects and methods

Patients and study design

A total of 115 HD patients (56 males; median age 47 years, range 16–89) in three dialysis centres in the city of Curitiba (Paraná-Brazil) were enrolled in the study. The main inclusion criterion was at least 6 months of HD treatment. Patients with chronic inflammatory disease (i.e. rheumatic diseases) and active infection, as well as hepatitis B, defined by the serum detection of HBs antigen, were excluded from the study. No patient, at the time of the beginning of the study, had more than 300 ml day of RRF. The clinical and dialysis data are shown in Table 1. The causes of renal failure were: chronic glomerulonephritis (n = 54), hypertensive nephrosclerosis (n = 32), diabetic nephropathy (n = 13) and other causes (n = 16). All patients were haemodialysed three times weekly with modified cellulosic membranes (cellulose acetate or derivatized cellulose).

The mean dose of i.v. iron therapy (weekly mean maintenance dose of 50–100 mg), and subcutaneous EPO (mean dose of 75 U/kg, range 50–100) was recorded during 6 months preceding the beginning of the study. The patients were divided by tertiles according to their ferritin levels and active infection, as well as hepatitis B, defined by the serum detection of HBs antigen, were excluded from the study. No patient, at the time of the beginning of the study, had more than 300 ml day of RRF. The clinical and dialysis data are shown in Table 1. The causes of renal failure were: chronic glomerulonephritis (n = 54), hypertensive nephrosclerosis (n = 32), diabetic nephropathy (n = 13) and other causes (n = 16). All patients were haemodialysed three times weekly with modified cellulosic membranes (cellulose acetate or derivatized cellulose).

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Table 1. Clinical and biochemical characteristics in 115 HD patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HCV+ group</th>
<th>HCV− group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)a</td>
<td>45 ± 11</td>
<td>49 ± 15</td>
<td>0.06</td>
</tr>
<tr>
<td>Prevalence of malesb</td>
<td>48</td>
<td>47</td>
<td>0.82</td>
</tr>
<tr>
<td>Time on HD (years)c</td>
<td>6.2 (0.7–12.6)</td>
<td>3.1 (1.1–15.4)</td>
<td>0.004</td>
</tr>
<tr>
<td>S-Alb (g/l)d</td>
<td>35 ± 3</td>
<td>35 ± 4</td>
<td>0.69</td>
</tr>
<tr>
<td>ALT (UI/l)e</td>
<td>23 (1–82)</td>
<td>14 (4–133)</td>
<td>0.0004</td>
</tr>
<tr>
<td>hsCRP (mg/l) f</td>
<td>3.3 (0.2–62)</td>
<td>4.8 (0.3–150)</td>
<td>0.09</td>
</tr>
<tr>
<td>IL-6 (pg/ml) f</td>
<td>4.6 (0.9–19.0)</td>
<td>4.2 (0.98–19.6)</td>
<td>0.42</td>
</tr>
<tr>
<td>Plasma pentosidine (pmol/mg albumin)</td>
<td>105 (13-233)</td>
<td>87 (26-184)</td>
<td>0.46</td>
</tr>
<tr>
<td>Ferritin (ng/ml)g</td>
<td>555 (16–1500)</td>
<td>483 (54–1500)</td>
<td>0.46</td>
</tr>
<tr>
<td>Malnutrition (SGA = B or C)h</td>
<td>46</td>
<td>54</td>
<td>0.08</td>
</tr>
</tbody>
</table>

aExpressed as means ± SD and statistical significance was tested with Student’s t-test.
bStatistical significance was tested with χ² test.
cExpressed as median and range and statistical significance was tested with non-parametric Mann-Whitney test.

Biochemical analysis

The HD patients were investigated on a mid-week day, before a dialysis session. Venous blood samples were collected from the HD patients in the morning after an overnight fast. Plasma samples were separated from blood cells and stored at −70°C pending analyses. Serum albumin (S-Alb) was determined by the bromcresol green method. High-sensitivity C-reactive protein (hsCRP) and alanine aminotransferase (ALT) were measured in the plasma by the nephelometry method. Plasma interleukin-6 (IL-6) levels were measured by an enzyme-linked immunosorbent assay (ELISA; Orthodiagnostic System, Raritan, NJ). Serum ferritin was measured by the Immulite method. The diagnosis of HCV was done by the detection of anti-HCV antibodies by a second-generation ELISA (ELISA-2, anti-HCV test; Orthodiagnostic System) based on six consecutive measurements performed monthly before the initiation of this study, and no seroconversion was observed during this period.

Pentosidine measurements

Plasma pentosidine was determined by reverse-phase HPLC as described originally by Odetti et al. [8] and modified by Miyata et al. [9]. Briefly, 50 μl of plasma were lyophilized and then hydrolysed by 50 μl of 6 M HCl at 110°C under a nitrogen atmosphere for 16 h, subsequently neutralized with 100 μl of 5 M NaOH and 200 μl of 0.5 M phosphate buffer (pH 7.4), then filtered through a 0.45 μm Millipore filter and diluted 20-fold with phosphate-buffered saline. Filtered samples (50 μl) were then injected into a C18 reverse-phase analytical column (218TPI04, Vydac, Separation Group, Hesperia, CA) using an on-line fluorescence detector at excitation/emission wavelengths of 335/385 nm. A linear solvent gradient was used as described by Wilker et al. [10], in which solvent A was 0.01 M heptfluorobutyric acid (HFBA) in water and solvent B was 60% acetonitrile + 40% H2O + 0.01 M HFBA. The elution profile was as follows: 0–3 min, 0% B; 3–20 min, 0–30% B; 20–25 min, 30% B; 25–35 min, 30–100% B; 36–45 min, 0% B linear gradient. The flow rate was maintained at 1 ml through the chromatographic run. Synthetic pentosidine was used for calculation of plasma levels. Since plasma pentosidine is mainly present as the protein-bound form and albumin is the only protein linking pentosidine [9], and free pentosidine represents 3–4% of the total circulating pentosidine, the plasma pentosidine concentrations in pmol/l were, therefore, corrected for serum albumin and expressed as pmol/mg of albumin.

Statistical analysis

Data are presented as mean ± SD, or median and range as appropriate. A P-value below 0.05 was considered to be significant. A comparison between two groups was performed using the Student’s t-test for normally distributed variables, whereas the Mann-Whitney U-test was used for non-normally distributed variables. Comparisons among the groups were made by one-way analysis of variance (ANOVA). We used the χ² test for categorical variables. For non-normally distributed variables, correlations were performed with the Spearman rank tests. A backward multiple stepwise regression analysis was used to assess the independent predictors of pentosidine levels.
Results

Clinical data and correlations

The clinical characteristics of the patients are given in Table 1. As expected, a significant negative correlation was found between S-Alb and hsCRP ($\rho = -0.21; P = 0.02$), and S-Alb and malnutrition (SGA) ($\rho = -0.21; P = 0.03$). Plasma pentosidine correlated with age ($\rho = 0.22; P = 0.001$) and ferritin ($\rho = 0.19; P = 0.03$). A positive correlation was found between IL-6 and age ($\rho = 0.38; P < 0.0001$) and between IL-6 and hsCRP ($\rho = 0.48; P < 0.0001$), and an inverse correlation was observed between IL-6 and S-Alb ($\rho = -0.29; P = 0.004$). The cumulative dose of iron was $776 \pm 498 \text{ mg}$ in the 6 months preceding the evaluation. The cumulative dose of iron correlated with haematocrit (Hct; $r = 0.38; P < 0.0001$) and ferritin ($r = 0.45; P = 0.02$). Finally, a significant correlation was found between ferritin and Hct ($\rho = 0.23; P = 0.001$), but no significant correlations were found between ferritin and hsCRP, IL-6 and ALT, respectively. A stepwise backward multivariate regression analysis including age, gender, malnutrition, CRP, HCV serology, ferritin, time on dialysis and iron dose as independent variables and pentosidine as a dependent variable showed that age and HCV were independent predictors of pentosidine levels ($P < 0.05; r^2 = 0.07$).

Comparison between patients according to HCV serology

The basal clinical and laboratory characteristics of the two groups, HCV+ ($n = 62$) and HCV− ($n = 53$), respectively, are given in Table 1. The time on HD treatment was significantly increased in the HCV+ compared with the HCV− patients. The HCV+ patients had significantly higher levels of ALT and plasma pentosidine than the HCV− patients (Table 1). The median level of serum hsCRP (mg/l) was lower in the HCV− than in the HCV+ group, but this difference was not statistically significant ($P = 0.07$). Finally, there was no difference in the levels of IL-6 between the two groups.

Comparison among patients according to ferritin tertiles

The patients were subdivided in tertiles according to the serum levels of ferritin (Table 2): group 1 ($n = 39$; ferritin $< 380 \text{ ng/ml}$), group 2 ($n = 37$; ferritin $380–750 \text{ ng/ml}$) and group 3 ($n = 39$; ferritin $> 750 \text{ ng/ml}$). The patients in group 3 had significantly higher median levels of pentosidine compared with group 1. Furthermore, the dose of iron was significantly higher in group 3 compared with group 1. On the other hand, age, time on HD, S-Alb, hsCRP, IL-6 and the presence of HCV were not significantly different among the three groups.

Discussion

The main findings of this study suggest that the presence of HCV was associated with higher plasma pentosidine levels, possibly indicating increased oxidative stress in this group of patients. To the best of our knowledge, our study is the first to report a correlation between HCV infection and increased levels of AGEs, specifically pentosidine. Under oxidative stress, the formation of AGEs such as pentosidine is the result of increased formation of reactive carbonyl compounds, formed by the autooxidation of carbohydrates and lipids [11].

Patients on HD are an important risk group for HCV infection. The prevalence of HCV in HD patients in Brazil differs from one region to another, from 20 to 50% [4]. HCV infection stimulates the production of reactive oxygen species by activated macrophages, and reactive aldehydes can directly activate hepatocytes, transforming them into myofibroblasts, thereby leading to hepatic fibrosis and cirrhosis. However, there

| Table 2. Clinical and dialysis characteristics in 115 HD patients placed in three groups according to the tertiles of ferritin |
|---|---|---|---|---|
| Age (years) | 45 ± 12 | 49 ± 14 | 47 ± 14 | 0.44 |
| Prevalence of males (%) | 65 | 37 | 41 | 0.02 |
| Time on HD (years) | 3.9 (0.7–9.1) | 4.7 (1.1–15.5) | 4.8 (0.9–12.4) | 0.21 |
| S-Alb (g/l) | 35.4 ± 3.7 | 34.9 ± 4.1 | 36.5 ± 5.3 | 0.41 |
| hsCRP (mg/l) | 4.8 (0.2–54) | 3.6 (0.2–150) | 3.3 (0.2–62.7) | 0.38 |
| IL-6 (pg/ml) | 4.6 (0.9–19) | 4.8 (0.9–18) | 4.2 (1.2–19) | 0.77 |
| Pentosidine (pmol/mg albumin) | 79 (13–177) | 99 (43–175) | 106 (27–230) | 0.03 |
| HCV (%) | 55 | 45 | 56 | 0.53 |
| Iron dose (mg/6 months) | 400 (100–1900) | 400 (100–3100) | 900 (300–2500) | 0.02 |
| ALT | 14 (4–133) | 17 (4–75) | 21 (1–96) | 0.64 |
| Hct (%) | 31 (14–46) | 34 (20–50) | 36 (20–51) | 0.03 |
| Malnutrition (SGA = B or C) (%) | 54 | 51 | 56 | 0.90 |

*Expressed as means ± SD and statistical significance was tested with parametric ANOVA.
**Statistical significance was tested with $\chi^2$ test.
***Expressed as median and range and statistical significance was tested with non-parametric ANOVA Kruskal–Wallis test.
Increased pentosidine in HD patients with HCV and hyperferritinaemia

is a lack of studies on the role of HCV on oxidative stress in the HD population. Köken et al. [12] studied the levels of malondialdehyde and carbonyl in a cohort of HD patients, and found that HCV+ patients presented higher levels of these oxidative stress markers compared with both controls and HCV− HD patients.

HCV infection may result in increased liver iron accumulation. The increase in iron stores may be due to release from damaged hepatocytes, action by the virus itself or genetic factors [13]. Furthermore, persistent HCV infection recently has been associated with atherosclerosis. Ishizaki et al. [14] demonstrated that HCV infection in non-renal patients, detected by the presence of HCV core protein, was significantly associated with the presence of carotid artery plaque (diagnosed by high-resolution B-mode ultrasonography).

Most HD patients require iron supplements to replace the continuous blood losses caused by the HD procedure. The absolute or relative iron deficiency, from the exhaustion of marrow iron stores to deliver adequate iron, is a common cause of failure of the EPO response [15]. Therefore, the administration of iron is a prerequisite for an adequate erythropoiesis. The safety and the side effects of i.v. iron have been debated in the past few years. Feldman et al. [7] evaluating the impact of parenteral iron administration on the survival and rate of hospitalization among 5833 HD patients in the USA showed an elevated risk of mortality in patients receiving >1000 mg of iron dextran over a period of 6 months. Moreover, i.v. iron administered to HD patients to correct anaemia can release free iron, which may react with hydrogen peroxide to produce the hydroxyl radical oxidants. The resulting oxiradicals have the potential to damage cellular lipids, nucleic acids, proteins and carbohydrates [16].

Although the average iron dose given to the patients followed the recommended weekly dose, ranging from to 50 to 100 mg, it should be noted that the level of ferritin in some patients exceeded the recommended upper limit in current guidelines. This can be explained by the fact that ferritin levels were measured during i.v. iron therapy, not monthly but every 3 months. Increased levels of ferritin, in the current study, were associated with the iron replacement therapy. Furthermore, in the present study, there was a significant correlation between the cumulative iron dose, ferritin and Hct, but not between ferritin and CRP and IL-6, respectively indicating that ferritin, in the current study, seemed to be a marker of iron stores rather than an inflammatory parameter. Moreover, the finding that the group of patients who presented with the highest levels of ferritin had the highest levels of pentosidine may indicate a possible association between i.v. iron therapy and increased levels of pentosidine.

Our findings are in accordance with other reports. Lim et al. [17] studied 50 HD patients according to the levels of ferritin, and showed that patients with the highest levels of ferritin had the highest levels of plasma lipid peroxides. In addition, Tovbin et al. [16] found that i.v. iron was associated with an increase in a marker of protein oxidation, AOPPs (advanced oxidation protein products). Similarly, Drücke et al. [18] demonstrated a correlation between increased common carotid artery intima-media thickness with AOPP, plasma ferritin and cumulative annual i.v. iron doses, indicating a possible role for i.v. iron therapy in oxidative stress. Furthermore, Kato et al. [19] reported that patients with HCV infection and iron loading had significantly higher levels of thioredoxin, indicating that oxidative stress may be aggravated by these two conditions. It was suggested recently that iron treatment should be carefully administered in HD patients with HCV, because of changes in iron metabolism in HCV [20]. Furthermore, haemoglobin and Hct levels have been reported to be higher in HCV+ than in HCV− end-stage renal disease (ESRD) patients [21]. Altogether, these findings suggest the need for a cautious approach in i.v. iron therapy in ESRD patients with HCV.

Some shortcomings of the current study should be considered. First, the diagnosis of HCV infection was based on an indirect test. Despite the fact that HCV+ patients showed higher levels of ALT, suggesting the presence of chronic infection, the presence of anti-HCV negativity with HCV RNA positivity is not rare in HD patients [22]. Secondly, despite the fact that ferritin was not correlated with the other two inflammatory parameters (IL-6 and CRP) in this study, and no significant correlation was found between ferritin and ALT levels, ferritin levels are known to be affected by inflammation and liver disease. Indeed, liver damage is known to weaken markedly the correlation between iron stores and ferritin level as ferritin is released into the plasma from damaged hepatocytes, independently of iron stores [23]. Finally, histological evaluation by liver biopsy could have better characterized the extent of liver injury and its association with HCV, ferritin and pentosidine.

In summary, the results of this study suggest that oxidative stress, as reflected by elevated levels of plasma pentosidine, was increased in patients with serological evidence of HCV infection, in particular in patients in the highest tertile of ferritin. These results suggest that oxidative stress might be enhanced in HD patients with a chronic infection influencing the liver, and that elevated ferritin levels due in part to iron overload, caused by i.v. iron therapy, may perhaps enhance this effect. Further studies are necessary to confirm the possible relationships between HCV, i.v. iron therapy and oxidative stress in HD patients.

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Conflict of interest statement. Bengt Lindholm is employed by Baxter Healthcare.
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