Plasma hydroxy metronidazole/metronidazole ratio in anti-HCV carriers with and without apparent liver disease

C. M. F. da Silva,¹ F. L. David,¹ M. N. Muscará,¹ S. S. Sousa,¹ J. G. P. Ferraz,² G. de Nucci,³ N. C. Polimeno⁴ & J. Pedrazzoli Jr¹

¹Clinical Pharmacology Unit, São Francisco University Medical School, Bragança Paulista, SP, ²Discipline of Clinical Gastroenterology, Department of Internal Medicine, Faculty of Medical Sciences, UNICAMP, Campinas, SP, ³Department of Pharmacology, Institute of Biomedical Sciences, São Paulo University, São Paulo, SP, and ⁴Hemocentro, São Francisco University Medical School, Bragança Paulista, SP, Brazil

Aims To evaluate plasma hydroxy-metronidazole/metronidazole ratio as a dynamic liver function test in HCV-infected individuals with/without liver disease, in the absence of liver cirrhosis.

Methods Metronidazole was administered intravenously in healthy volunteers, asymptomatic anti-HCV-positive blood donors, and in chronic hepatitis C patients. Serology to HCV was determined by a second generation assay and confirmed by gelatin particle agglutination test using recombinant antigens C22-3 and C200. Plasma concentration of metronidazole and hydroxy-metronidazole was measured by high performance liquid chromatography in samples collected 5, 10, 20 and 30 min following the end of metronidazole infusion.

Results Chronic hepatitis C patients had abnormal liver enzymes, while healthy volunteers and anti-HCV-positive blood donors had normal liver biochemistry tests. Plasma metronidazole concentration was similar in all groups studied. Plasma hydroxy-metronidazole/metronidazole ratio was significantly reduced in HCV-infected subjects, an effect observed 10 min after the end of drug infusion.

Conclusions Metronidazole clearance is impaired in anti-HCV-positive blood donors and chronic hepatitis C patients, indicating that HCV is capable of affecting liver function at early stages of the disease. The metronidazole clearance test can detect impaired liver function in HCV-infected individuals even in the absence of liver cirrhosis.

Keywords: metronidazole clearance, liver function, hepatitis C, cytochrome P450, blood donors

Introduction

Hepatitis C virus (HCV) is the predominant cause of post-transfusion and sporadic non-A, non-B hepatitis worldwide [1]. Subclinical HCV infection is common, without patients reporting the acute illness associated with jaundice [2]. Although the acute illness is usually mild, the most important feature of HCV infection is its tendency to become chronic, with increased risk of developing cirrhosis and hepatocellular carcinoma [3, 4]. It has been suggested that persistence of HCV infection is invariably associated with hepatic damage [5]. However, there have been reports of anti-HCV positive individuals in which persistent HCV viraemia was not associated with abnormal liver chemistry [6]. Therefore, considerable controversy exists regarding the existence of a true healthy HCV carrier state.

Dynamic liver function tests such as indocyanine green clearance [7], caffeine elimination [8] and lignocaine

metabolism [9] have been used to complement standard liver assessment in patients with liver disease. Metronidazole clearance was recently proposed as a very sensitive method for evaluating liver function [10]. It consists of measuring the plasma hydroxy-metronidazole/metronidazole ratio by high performance liquid chromatography (h.p.l.c.) following intravenous administration of a single dose of metronidazole. It assessed a decreased capacity of Child A cirrhotic patients to metabolize metronidazole compared with healthy individuals, indicating that detection of impaired liver function is possible at very early stages of liver cirrhosis [10].

Since the significance of anti-HCV positivity in asymptomatic blood donors is unclear with respect to liver disease, and the infection rate in this group can be as high as 20% [11], evaluation of liver function in these individuals might prove useful in detecting subgroups of patients that could potentially develop liver injury. Therefore, the aim of this study was to evaluate the hepatic clearance of metronidazole in healthy anti-HCV positive blood donors, chronic HCV-hepatitis patients and in healthy volunteers. Using the plasma hydroxy-metronidazole/metronidazole ratio as a liver

Correspondence: Professor Dr José Pedrazzoli Júnior, Clinical Pharmacology Unit, São Francisco University Medical School, Av. São Francisco de Assis, 218 12900-000, Bragança Paulista, SP, Brazil.

function test [10] we have assessed the impact of HCV infection on liver function in the absence of cirrhosis.

Methods

Subjects

Three groups of subjects were studied: healthy volunteers, asymptomatic anti-HCV positive blood donors and patients with HCV-associated chronic hepatitis. All individuals gave their written informed consent prior to entering the study. Patients diagnosed with liver tumors and/or those using drugs which could influence cytochrome P450 function were excluded from the study. The clinical protocol was approved by the Medical School Ethics Committee, and the study was conducted in accordance with the Declaration of Helsinki.

The healthy volunteers (males = 12, females = 3; median age = 32 years [range 19–48 years]; median weight = 72 kg [range 52–96 kg]) were determined following assessment of their medical history, absence of alcoholism, normal physical examination and laboratory tests, including negative serology for B and C hepatitis. Sixteen healthy, asymptomatic, consecutive anti-HCV positive blood donors (males = 14, females = 2; median age = 35 years [range 18–76 years]; median weight = 67 kg [range 45–74 kg]) were referred to the Gastroenterology Unit of the São Francisco University Hospital between 1993 and 1996 from the São Francisco University Hospital Blood Bank (Hemocentro-HUSF). They were tested for anti-HCV antibodies using a second generation enzyme immunoassay (ABBOT HCV EIA 2nd Generation, ABBOTT Laboratories Diagnostics Division, IL). All positive results were confirmed using a gelatin particle agglutination test with recombinant antigens C22-3 and C200 (Serodia-HCV, Fujirebio Inc., Japan). Anti-HCV-positive blood donors were considered as healthy carriers if liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase and γ -glutamyl transpeptidase, were within the normal reference range values. Each patient was tested in three different occasions with at least 1 month interval between tests. Absence of cirrhosis was determined by clinical data together with normal abdominal ultrasonography.

Thirteen non-alcoholic, non-cirrhotic, HCV-associated chronic hepatitis patients (males = 13; median age = 34 years [range 21–47 years]; median weight = 68 kg [range 50–113 kg]) were selected as having positive serum anti-HCV (as measured by EIA), persistently abnormal liver enzymes but absence of other forms of viral hepatitis (negative serology for HBV). Absence of liver cirrhosis and chronic HCV hepatitis were confirmed histologically by percutaneous liver biopsy. Two patients from this group refused this procedure, and therefore absence of cirrhosis was determined by clinical evaluation and ultrasonography.

Clinical laboratory analysis

Serum AST, ALT, alkaline phosphatase, γ -glutamyl transpeptidase, total bilirubin, prothrombin time, haematocrit and total/differential white cell counts were analyzed in all subjects.

Metronidazole metabolism rate determination

Metronidazole clearance test was performed as described previously [10]. Briefly, metronidazole (Flagyl[®], 5 mg ml⁻¹; from Rhodia, SP, Brazil) was administered intravenously as a single infusion (100 ml) over 20 min. Blood samples (5 ml) from an antecubital vein were collected into EDTA-containing tubes before and 5, 10, 20 and 30 min following the end of drug infusion. The participants remained as outpatients at the Clinical Pharmacology Unit during the metronidazole administration and blood sampling. The blood samples were centrifuged at 2000 g for 5 min and the plasma was separated and stored at -20°C until assayed for their metronidazole (MET) and the hydroxy metabolite (OHM) concentration by h.p.l.c., as previously described [10, 12]. Individual MET and OHM concentration, as well as OHM/MET concentration ratio, was calculated and plotted as a function of time.

Statistical analysis

The results were expressed as mean $\pm$ s.d., unless otherwise stated. All the variables were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons among groups. Differences were considered significant when $P < 0.05$.

Results

Blood analysis demonstrated that both healthy volunteers and asymptomatic anti-HCV-positive blood donors had liver enzyme levels, total bilirubin and prothrombin time within the reference values (Table 1). However, chronic hepatitis C patients had significantly elevated liver enzyme levels compared with both healthy volunteers and anti-HCV-positive blood donors, indicating that this group of subjects had active liver disease (Table 1). However, these patients had normal total serum bilirubin and prothrombin time (Table 1). Alkaline phosphatase, hematocrit and total/

Table 1 Results of liver biochemistry tests from the three groups of subjects studied. There were no differences in total bilirubin and prothrombin time among the groups studied, while chronic hepatitis C patients had abnormal liver enzymes compared with healthy individuals and asymptomatic anti-HCV-positive blood donors. Asterisks denote significant differences compared with healthy volunteers (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

	Healthy volunteers	Anti-HCV+ blood donors	Chronic HCV patients
AST (IU ml ⁻¹)	17.1 $\pm$ 10.3	18.7 $\pm$ 3.6	72.3 $\pm$ 55.2***
normal (12–46 IU ml ⁻¹)			
ALT (IU ml ⁻¹)	16.4 $\pm$ 9.6	19.3 $\pm$ 5.2	137.1 $\pm$ 141.5**
normal (3–50 IU ml ⁻¹)			
γ -GT (IU ml ⁻¹)	22.2 $\pm$ 15.8	17.1 $\pm$ 10.6	57.5 $\pm$ 0.5***
normal (6–28 IU ml ⁻¹)			
Total bilirubin (mg %)	0.88 $\pm$ 1.6	0.90 $\pm$ 0.7	1.15 $\pm$ 1.0
normal (<1.0 mg %)			
Prothrombin time (s)	13.5 $\pm$ 0.5	12.8 $\pm$ 0.2	12.9 $\pm$ 0.3
control (13.5 s)			

differential white cell count were within the normal reference range in all subjects studied (data not shown).

The rise in plasma metronidazole concentration was similar in all groups studied following metronidazole infusion (Figure 1a), whilst plasma hydroxy-metronidazole concentration was significantly reduced in both anti-HCV-positive blood donors and chronic hepatitis C patients (Figure 1b). Assessment of plasma hydroxy-metronidazole/metronidazole ratio (Figure 1c), an index of liver function, demonstrated a

significantly reduced capacity of anti-HCV-positive blood donors and chronic hepatitis C patients to metabolize intravenously infused metronidazole compared with healthy individuals, a phenomenon observed in samples collected 10 min following the end of metronidazole infusion (healthy volunteers = 0.069 ± 0.029 ; anti-HCV-positive blood donors = 0.044 ± 0.019 , CI -0.041 – 0.0085 , $P < 0.01$; chronic hepatitis C patients = 0.033 ± 0.013 , CI -0.057 – 0.015 , $P < 0.001$). This effect was also observed in samples collected at further time points.

Discussion

Standard liver biochemistry tests are not useful for assessing hepatic function or predicting development of cirrhosis in chronic hepatitis patients [13]. Although approximately 95% of HCV-infected individuals can be identified by second generation anti-HCV testing (EIA), this test is not useful in discriminating previous and presently active infection [14]. Moreover, HCV is not always associated with liver injury, since some HCV RNA-positive subjects have been described as having normal liver histology [6] and normal liver biochemistry tests [15]. Further complicating this issue, it has been reported that individuals with detectable serum HCV RNA have indeed some degree of liver damage, despite producing normal liver enzymes [5, 16]. Therefore, HCV-infected individuals can present with: 1) normal liver enzymes/normal liver histology, 2) abnormal liver enzymes/normal histology, 3) normal liver enzymes/abnormal liver histology, and 4) abnormal liver enzymes/abnormal liver histology.

The development of HCV-associated cirrhosis is related to poor prognosis. An investigation of the factors that could predict which subset of HCV-infected patients will develop a more severe degree of liver injury in the future is required. Some previous studies have attempted to associate different HCV genotypes to long-term effects on liver histology [17–19]. However, the effects of HCV infection on liver function have not been fully investigated. The present study evaluated the impact of HCV on metronidazole clearance in the absence of liver cirrhosis. We have previously demonstrated the diagnostic value of metronidazole clearance as a very sensitive, easy to perform, liver function test. This test is capable of discriminating Child A cirrhotic patients from healthy individuals [10]. Our present results indicate that metronidazole metabolism, as measured by the plasma hydroxy-metronidazole/metronidazole ratio following metronidazole infusion, is progressively impaired in anti HCV-positive blood donors and in chronic hepatitis patients when compared with healthy volunteers. These results suggest that HCV can determine significant abnormalities on liver function.

Metronidazole is extensively metabolized by, as yet, unidentified cytochrome P450 isozyme(s) [20], giving rise to two principal metabolites: the hydroxy metabolite (with about 65% of the pharmacological activity of metronidazole), and the inactive acetic acid metabolite [21]. Several viral and bacterial infections [22], as well as interferon-inducing agents [23] have been associated with impaired cytochrome P450-mediated drug metabolism. This has been attributed, at least in part, to cytokine release, since interleukin-2 [24],

Figure 1 Mean $\pm$ s.e. mean plasma concentration vs time curves obtained for plasma MET (panel a), OHM (panel b) and OHM/MET ratio (panel c) from the three groups of subjects studied: healthy volunteers (●), anti-HCV-positive blood donors (○) and chronic C-virus hepatitis patients (△). Asterisks denote significant difference compared with healthy volunteers (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

interleukin-4 [25] and interferon gamma have been shown to down-regulate the expression and catalytic activities of cytochrome P450 enzymes and genes [26, 27]. Indeed, elevated levels of interleukin-2, interleukin-4, interleukin-10 and interferon gamma have been reported in chronic hepatitis C [28, 29]. Therefore, it is probable that an impaired P450-dependent metronidazole metabolism exists in HCV-infected patients.

The mechanisms involved in hepatocellular injury in chronic HCV infection remain unclear. HCV infection may affect the hepatocytes directly or as result of an abnormal immune response within the liver [30, 31]. HCV-related factors can potentially produce cytopathic effects, and are dependent on its replication and mutation rates, as well as by the infectious dose. The immune response is dependent on host factors, including age and maturity of the immune system, local and systemic cytokine production, and humoral and cellular immune response [32]. That the direct effects of HCV infection on hepatocytes is unlikely is supported by the observation that: a) a percentage of individuals with antibodies to HCV and HCV RNA positivity have normal liver enzymes with no (or minimal) histological findings; b) intrahepatic HCV RNA levels do not correlate with the degree of liver injury; and c) HCV healthy carriers and chronic hepatitis patients have similar virological characteristics [14, 33]. On the other hand, a role for anti-HCV immune mediated liver cell damage has been suggested by immunohistochemical studies [34]. It is believed that HCV is able to trigger aberrant autoreactivity to hepatocellular antigens [35–39]. Moreover, autoantibodies produced during chronic hepatitis C are capable of recognizing several forms of P450 cytochrome enzymes described in the plasma membrane of human hepatocytes, the specific content of cytochrome P450 being 9% of that in microsomes. Such P450 cytochromes are functional and were recognized by anti-cytochrome P450 autoantibodies [40–43]. Once again, this data raises the possibility of an autoimmunity-mediated interference with the metronidazole metabolizing cytochrome P450 enzyme in HCV-infected subjects.

We conclude that anti-HCV-positive healthy blood donors and chronic hepatitis C patients have impaired liver function, as determined by altered metronidazole metabolism. HCV infection can determine changes in liver function at early stages of the disease. This phenomenon does not seem to be associated with damaged hepatocytes, and we speculate that it is most likely related to cytokine-mediated down-regulation of cytochrome P450 and/or an autoimmune-mediated impairment of cytochrome P450 function. This suggests that HCV-infected individuals may have impaired cytochrome P450-dependent drug metabolism. The use of metronidazole clearance test in the follow-up of HCV-infected subjects might help in detecting those patients who may develop more severe degrees of liver injury.

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