CSYKINES AND LIPID PEROXIDATION IN ALCOHOLICS WITH CHRONIC HEPATITIS C VIRUS INFECTION

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Abstract — A major cause of liver cirrhosis and hepatocarcinoma is chronic infection by hepatitis C virus. Ethanol consumption is the most significant environmental factor that exacerbates the progression of chronic hepatitis C to liver cirrhosis and hepatocarcinoma, perhaps due to increased cytokine secretion together with increased lipid peroxidation. In this study, we compare the intensity of lipid peroxidation (estimated as malondialdehyde (MDA) serum levels), the antioxidant status (measured as glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities in red blood cells), and levels of cytokines derived from Th1 cells (such as interferon gamma (IFNG)), Th2 cells (such as interleukin (IL)-4), Th3 cells (such as transforming growth factor beta (TGF-β)), and IL-6, IL-8, and tumor necrosis factor (TNF-α) in patients affected by chronic hepatitis C virus infection, 26 drinkers of alcohol and 40 nondrinkers of alcohol. Patients showed significantly higher TNF-α (Z = 4.92, P < 0.001), IL-8 (Z = 4.95, P < 0.001), IFNG (Z = 2.81, P = 0.005), TGF-β (t = 2.12, P = 0.037), MDA (Z = 5, P < 0.001), but lower IL-6 (Z = 3.61, P < 0.001) and GPX (F = 4.30, P < 0.05) than controls, whereas no differences were observed regarding IL-4 (Z = 0.35, P = 0.72), GPX and SOD activities. Alcoholics showed significantly higher TNF-α, but lower IL-4, MDA, and GPX, than nonalcoholics. TNF-α was significantly related to albumin and prothrombin activity, whereas TGF-β was significantly related to MDA levels. Thus, cytokine secretion is altered in HCV infection. This alteration mainly consists of a stimulation of Th1 cytokines and an inhibition—or at least, no stimulation—of Th2 cytokines; these changes are especially marked among alcoholics with HCV infection, and are accompanied by raised TGF-β.

INTRODUCTION

A major cause of liver cirrhosis and hepatocarcinoma is chronic infection by hepatitis C virus (HCV). This frequent infection affects at least 1.5–3% of the population worldwide (Chevaliez and Pawlotsky, 2007), but only a minority develop chronic liver disease and, eventually, hepatocarcinoma (Yano et al., 1993; Takahashi et al., 1993; Tong et al., 1995). The factors involved in the progression to chronic forms of liver disease are not completely known, but epidemiological, clinical, and histological evidences suggest that ethanol consumption is the most significant environmental factor that exacerbates the progression of chronic hepatitis C (Poynard et al., 1997; Wiley et al., 1998). Several pathways may be involved in this enhancing effect, including modification of the immune response and cytokine secretion. Different subsets of activated T cells secrete different cytokines, so IFNG represents the main cytokine derived from Th1 activation, IL-4 the main cytokine derived from Th2 activation, and transforming growth factor beta (TGF-β), the main cytokine derived from the so-called Th3 and Tr1 cells (Taylor et al., 2006). In addition, potent pleiotropic cytokines, such as IL-6 and tumor necrosis factor alpha (TNF-α), ascribed to the Th2 and Th1 subsets, respectively, exert proinflammatory effects, creating a positive feedback loop. Secretion of all these cytokines is related, in diverse forms, to lipid peroxidation, liver cell damage, antioxidant activity, and increased progression of fibrosis in the liver. Although several authors have reported altered cytokine levels in chronic HCV infection, with diverse results (Nelson et al., 1997; Fan et al., 1998; Neuman et al., 2002; Spanakis et al., 2002, among others) the cytokine pattern in patients with HCV infection, with or without simultaneous ethanol consumption, and its relationship with oxidative stress and liver histological changes, is not well known.

The purpose of this study is to compare, based on these facts, the intensity of malondialdehyde (MDA) production, the antioxidant status (measured as glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities in red blood cells), and levels of cytokines derived from Th1 cells (such as IFNG), Th2 cells (such as IL-4), Th3 cells (such as TGF-β), and IL-6, IL-8, and TNF-α in patients affected by chronic HCV infection, drinkers of alcohol, and nondrinkers of alcohol.

METHODS

Patients and controls

We have studied a cohort of 66 consecutive patients affected by hepatitis C virus infection, who were electively hospitalized (from July 2004 to December 2005) in the Internal Medicine unit for programmed liver biopsy. All these patients had been previously admitted to our unit, either by decompensated liver disease, infection, or other diseases, and were offered a treatment with ribavirin and interferon, provided cessation of alcohol intake. During that admission, patients were classified according to ethanol intake in alcoholics (alcoholic men drinking more than 80 g ethanol per day and alcoholic women drinking more than 40 g ethanol per day) and nonalcoholics (drinking less than 10 g/day). Indeed, some of them (26) were heavy drinkers (of more than 200 g/day), whereas others did not drink at all or drank only sporadically (less than 10 g ethanol/day). When the alcoholics were admitted for biopsy, ethanol intake was reduced strongly by most

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Table 1. Some biological parameters of patients and controls (x ± SD are shown; in cases of non-normal distribution, we also provide median and interquartile range)

<table>
<thead>
<tr>
<th></th>
<th>Alcohol + hepatitis C</th>
<th>Hepatitis C without alcohol</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of individuals</td>
<td>26</td>
<td>40</td>
<td>39</td>
</tr>
<tr>
<td>Age (years)</td>
<td>40.7 ± 10.1</td>
<td>43.5 ± 8.3</td>
<td>41.28 ± 8.21</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.7 ± 2.6</td>
<td>23.7 ± 4.5</td>
<td>26.3 ± 3.4</td>
</tr>
<tr>
<td>Ethanol consumption (daily amount (g))</td>
<td>74 ± 47</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Serum albumin (g/l)</td>
<td>3.98 ± 0.50</td>
<td>4.03 ± 0.43</td>
<td>–</td>
</tr>
<tr>
<td>Prothrombin activity (%)</td>
<td>90.8 ± 12.2</td>
<td>96.9 ± 4.9</td>
<td>–</td>
</tr>
<tr>
<td>Serum bilirubin (mg/dl)</td>
<td>1.09 ± 0.15</td>
<td>0.70 ± 0.46</td>
<td>–</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>14.59 ± 1.97</td>
<td>14.37 ± 2.16</td>
<td>14.72 ± 1.17</td>
</tr>
<tr>
<td>Serum ASAT (U/l)</td>
<td>100 ± 103</td>
<td>73 ± 80</td>
<td>7–40*</td>
</tr>
<tr>
<td>Serum ALT (U/l)</td>
<td>67 (45–118)</td>
<td>51 (33–89)</td>
<td>–</td>
</tr>
<tr>
<td>Serum GGT (U/l)</td>
<td>127 ± 119</td>
<td>80 ± 50</td>
<td>7–40*</td>
</tr>
<tr>
<td>Knodell index</td>
<td>7.40 ± 2.53</td>
<td>6.73 ± 3.48</td>
<td>–</td>
</tr>
<tr>
<td>Cytosinosis (yes/total)</td>
<td>0/26</td>
<td>6/40</td>
<td>0/39</td>
</tr>
<tr>
<td>HIV coinfection (yes/total)</td>
<td>18/26</td>
<td>26/40</td>
<td>0/39</td>
</tr>
<tr>
<td>Men/Women</td>
<td>22/4</td>
<td>25/15</td>
<td>28/11</td>
</tr>
<tr>
<td>Genotype (1/no 1)</td>
<td>4/22</td>
<td>12/26</td>
<td>–</td>
</tr>
<tr>
<td>Viral load</td>
<td>1.68 ± 1.75</td>
<td>1.65 ± 3.18</td>
<td>–</td>
</tr>
<tr>
<td>(×10⁶ copies/ml)</td>
<td>1.07 (0.57–2.56)</td>
<td>0.77 (0.42–1.61)</td>
<td>–</td>
</tr>
</tbody>
</table>

*Normal range in our laboratory.

patients; data presented in Table 1 belong to this period. Ethanol intake was assessed by recall (Jarque-López et al., 2001) and calculated as follows: grams of ethanol = volume of beverage (ml) × strength (v/v, as %) × 0.8. Clinical characteristics of the patients are listed in Table 1. HCV infection was documented by the presence of anti-HCV antibodies, and viral load was determined by quantitative reverse transcription polymerase chain reaction. No differences in viral load were observed between alcoholic and nonalcoholic patients (Z = 0.86, P = 0.4).

The control group was composed of 39 healthy health workers, drinkers of less than 10 g ethanol day, aged 41.3 ± 8.2 years. Age differences between the three groups (drinkers, nondrinkers, and controls) were not statistically significant.

**Cytokines and biochemical parameters**

Blood samples were taken at 8.00 am in fasting conditions, and the liver biopsy was performed on the same day. Blood samples were immediately frozen at −80°C. The following parameters were determined.

**Cytokines.** TNF-α by immunometric chemiluminescence assay (intra-assay variation coefficient 4–6.5%, interassay variation coefficient 2.6–3.6%, recovery 92–112%), Diagnostic Products Corporation (DPC), Los Angeles, CA); IL-6, by chemiluminescence assay (intraassay variation coefficient 5.3–7.5%, recovery 85–104%, DPC, Los Angeles, CA); IL-4, by enzyme-linked immunosorbent assay (ELISA); (inter- and intra-assay coefficient of variation <10%; sensitivity <2 pg/ml; recovery 101%, Bender MedSystems Diagnostics GmbH, Vienna, Austria); IL-8, by chemiluminescence assay (intraassay variation coefficient 5.3–7.5%, DPC, Los Angeles, CA); interferon gamma (IFNG), by ELISA (inter- and intra-assay coefficient of variation 0.3–10.7%; sensitivity <1.5 pg/ml; recovery 90–112%; Bender MedSystems Diagnostics GmbH, Vienna, Austria). TGF-β, by ELISA (Immunobiological Laboratories, Hamburg, Germany); sensitivity 1.9 pg/ml.

**Lipid peroxidation products.** Serum MDA levels, referred to as thiobarbituric acid-reactive substance (TBARS), were measured according to the method described by Kikugawa et al. (1992). A volume sample of 0.2 ml of plasma was added to 0.2 ml of H₂PO₄ (0.2 M) and the color reaction was initiated by adding 25 µl of 0.11 M thiobarbituric acid (TBA) solution. Samples were placed in a 90°C heating block for 45 min. After the samples were cooled, the TBARS (pink complex color) were extracted with 0.4 ml of n-butanol. The butanol phase was separated by centrifugation at 6000 × g for 10 min. Aliquots of the n-butanol phase were placed in a 96-well plate and read at 535 nm with a microplate spectrophotometer reader (Benchmark Plus, Bio-Rad, Hercules, CA). The calibration curve was prepared with authentic MDA standards of 0–20 µM. The intra- and inter-assay variation coefficients were 1.82 and 4.01, respectively.

**Enzyme activities in red blood cells**

**Cu/Zn SOD activity.** Red blood cells were washed three times with an ice-cold isotonic sodium chloride solution. Hemolysis of the washed cells suspension was achieved by mixing 1 volume of cells with 4 volumes of ice-cold distilled water. The hemolysate was centrifuged at 13,000 × g for 15 min at 4°C. The assay was performed on the supernatant. The Cu/Zn SOD assay kit uses autooxidation of 5,6,6,11-tetrahydro-3,9,19-tri-hydrobenzofluorene, a chromophore that absorbs maximally at 525 nm, provided by Calbiochem (San Diego, CA). The activity was calculated directly from the rate of sample versus the average of blank control, using the ratio table provided in the Calbiochem kit.

**Selenium-dependent GPX activity.** We have used GPX assay to measure activity indirectly, using the oxidation of NADPH to NADP recording the absorbance at 340 nm (A₃₄₀) (Calbiochem, San Diego, CA). The reaction was initiated by adding tertbutyl hydroperoxide to a solution containing reduced glutathione, glutathione reductase, and NADPH, and the sample (containing GPX). The oxidation of NADPH to NADP was accompanied by a decrease in A₃₄₀. The rate of the decrease in absorbance is directly proportional to GPX activity in the sample. The assay was performed on the supernatant. The activity was calculated using the sample data and calculation sheet provided by the Calbiochem kit (1 µM/ml = 1 nmol NADPH/min/ml = A₃₄₀/min/0.0062).

In addition, patients underwent routine laboratory evaluation (Table 1). The study protocol was approved by the local ethical committee of our Hospital and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

**Statistics**

The Kolmogorov–Smirnov test was used to test normality, a condition not fulfilled by some of the cytokines analyzed. Therefore, nonparametric tests, such as Mann–Whitney U-test and Kruskall–Wallis, were used to analyze differences in these parameters between groups. Spearman’s correlation analysis was used to compare quantitative parameters. Some parameters were different among some subgroups (i.e., men/women, or HIV infected/noninfected patients). These subgroups also...
showed an association with ethanol consumption. Therefore, in order to detect independent effects and interactions, we performed a two-way variance analysis and, when interactions were observed, we performed a stepwise multiple correlation analysis to find out with which of the different subgroups a certain parameter was related significantly. All these analyses were performed with the aid of SPSS 14.0 (Statistical Package for Social Sciences, Chicago, IL).

RESULTS

The results are shown in Tables 2–4. Taking together drinkers and nondrinkers, patients showed significantly higher TNF-α (Z = 4.92, P < 0.001), IL-8 (Z = 4.95, P < 0.001), INFG (Z = 2.81, P = 0.005), and TGF-β (t = 2.12, P = 0.037), but lower IL-6 (Z = 3.61, P < 0.001) than controls, whereas no differences were observed in IL-4 (Z = 0.35, P = 0.72). One of the alcoholic patients showed a very high value of IL-6 (72.2 pg/ml). Differences regarding IL-6 were even more marked if this case was excluded from the analysis. Also, patients showed higher MDA values (Z = 5, P < 0.001), but no differences in GPX and SOD activities. In Table 2 we show median values of these parameters in alcoholics, nonalcoholics, and controls, as well as the results of the statistical analyses comparing the three groups (Kruskal–Wallis (KW) tests) or ANOVA (F values) for which independent samples were introduced in a two-way variance analysis. No differences were observed between men and women regarding the parameters analyzed in Table 3, nor between HIV infected and noninfected patients (Table 4). TNF-α (F = 12.64, P = 0.001) and IL-4 (F = 5.47, P = 0.024), but not INFG, MDA, and GPX, still showed statistically significant differences between alcoholics and nonalcoholics when sex and HIV infection were introduced in a two-way variance analysis. No interactions were observed between sex or HIV and any of the parameters analyzed regarding differences between alcoholics and nonalcoholics, except for TNF-α, for which independent effects of alcoholic liver disease (F = 12.64, P = 0.001), sex (F = 5.46, P = 0.023), and HIV (F = 0.83, P = 0.011), as well as an interaction between alcoholic liver disease and HIV (F = 4.74, P = 0.034), were observed. Therefore, we performed a stepwise multiple correlation analysis between TNF-α and presence or not of alcoholic liver disease, sex, and HIV infection. Only alcoholic liver disease showed an independent, significant relationship with TNF-α (β = 0.35, P = 0.004), IL-4 (β = 0.32, P = 0.023), and GPX (β = 0.26, P = 0.037).

Relationships with histological changes and liver function

Knodell index was clearly related to INFG (ρ = 0.41, P < 0.001) and IL-6 (ρ = 0.39, P = 0.002), but not to any of the remaining cytokines or viral load.
TNF-α was related to serum ALAT (ρ = 0.26, P = 0.035), albumin (ρ = −0.27, P = 0.042), bilirubin (ρ = 0.24, P = 0.05), and prothrombin activity (ρ = −0.31, P = 0.012). Albumin was directly related to TGF-β (ρ = 0.25, P = 0.043). IL-6 was also inversely related to serum albumin (ρ = −0.25, P = 0.042).

Relationships between cytokines, lipid peroxidation, and antioxidant enzymes
MDA levels were significantly related to IL-6 (ρ = 0.35, P = 0.004) and TGF b (ρ = 0.26, P = 0.033).
IFNG was related to IL-4 (ρ = 0.33, P = 0.018), IL-8 (ρ = 0.26, P = 0.033), and IL-6 (ρ = 0.27, P = 0.028).
TNF-α was inversely related to TGF-β (ρ = −0.34, P = 0.005).

No relation was observed between viral load and any of the cytokines analyzed.

DISCUSSION

In the present study patients with chronic HCV infection show higher TNF, IL-8, and IFNG levels, normal IL-4 and low IL-6 levels, and raised TGF-β levels, compared to a control group. Cytokines were determined in blood samples obtained when the patients were admitted for a programmed liver biopsy, so altered cytokine levels cannot be attributed to acute infection or other intercurrent diseases, and are probably solely related to the chronic HCV infection. In addition, our data are consistent with a more marked alteration of cytokine secretion among alcoholics, who showed higher IFNG, lower IL-4 (a Th2 cytokine), and higher TNF-α values than nonalcoholics. All these results are in agreement with the current knowledge relative to progression of chronic hepatitis C infection, in which several mechanisms are involved, such as modification of the immune response (Eggers et al., 2006), activation of proinflammatory cytokines both by virus C infection (Fan et al., 1998) and chronic alcoholism (Fujimoto et al., 2000; Sánchez-Pérez et al., 2006), synergistic oxidative stress (Kono et al., 2000; Okuda et al., 2002) and, debatably (Plumlee et al., 2005), increased viral replication.

The cytokine pattern observed in our study is consistent with a stimulation of Th1 and Th3 lymphocyte subsets, together with nonactivation of Th2 cells. Results regarding cytokine pattern in HCV infection are diverse. Several authors have documented raised Th1 and Th2 responses (Spanakis et al., 2002), with raised IL-6 levels, a result in contrast with that reported in this study, which, on the other hand, is in agreement with Lee et al. (2002), who also found low IL-6 levels; in a similar sense, Kowala-Piaskowska et al. (2004) report that IL-6 levels lack prognostic significance in children on which the prognostic assessment was made were HCV infected treated with interferon and ribavirin. Discordant results also exist regarding Th1 cytokines, such as IFNG. Whereas Fan et al. (1998) document normal IFNG and enhanced Th2 response, Trapero-Marugán et al. (2006) found that higher intracellular expression of IFNG was associated with sustained viral response, a result in contrast with that reported by Masaki et al. (2002), who found a lower Th1/Th2 population ratio in responders, but in agreement with Falleti et al. (2007), who observed that a high production of IFNG was associated with slower fibrosis progression in patients with recurrent hepatitis C after liver transplantation.

Rised TGF-β levels have been reported by the vast majority of authors (Nelson et al., 1997; Neuman et al., 2002), in relation to histological alterations such as fibrosis or Knodell index, respectively. TGF-β is a cytokine secreted by regulatory T cells and Th3 cells and is involved in immune suppression and antibody isotype regulation, skewing the IgE response to allergens toward an IgA response (Taylor et al., 2006). On the other hand, TGF-β is clearly related to liver fibrogenesis, not only in HCV-infected patients, but also in other forms of chronic liver disease (Chen, 2002; Parsons et al., 2007). Despite this fact, in this study we failed to find an association between TGF-β and histological changes, in agreement with some classic studies (Roulot et al., 1995). However, we did find a significant relationship between TGF-β and MDA levels, a finding consistent with the well-known relationship between inflammation, lipid peroxidation, and liver fibrosis, which has been widely documented in alcoholic liver disease (Bird et al., 1990; Crews et al., 2006; Sánchez-Pérez et al., 2006), nonalcoholic steatohepatitis (Torer et al., 2007), and also viral hepatitis C (Yadav et al., 2002; Levent et al., 2006) and B (Dikici et al., 2005). Moreover, Paradis et al. (1997) found a direct relationship between fibrosis and liver MDA in patients with HCV infection. In the same way, we interpret the relationship between TNF-α and liver function parameters, in agreement with the well-known relationship between TNF and liver inflammation (Yin et al., 1999; Neuman et al., 2002).

Chronic ethanol consumption leads to activation of cytochrome P-450 2E1, which constitutes a source of reactive oxygen species (ROS) (Otani et al., 2005), a factor that adds to the production of ROS induced by the expression of hepatitis C proteins (Okuda et al., 2002). In accordance with these statements, we found raised MDA levels in our patients, but they were higher in nonalcoholic HCV infected patients than in the alcoholic ones. MDA levels were estimated as TBARS. It is important to keep in mind that TBARS concentration not only reflects MDA levels, but also several other compounds derived from oxidation (Del Rio et al., 2005), so that

Table 4. Cytokine values and HCV viral load in HIV positive and HIV negative patients. Results are given as mean ± standard deviation and, in the second subfile, median (interquartile range).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>HIV positive (pg/ml)</th>
<th>HIV negative (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>24.91 ± 37.48</td>
<td>13.62 ± 9.09</td>
</tr>
<tr>
<td>TNF-α</td>
<td>12.46 ± 6.70</td>
<td>10.08 ± 4.72</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.26 ± 10.64</td>
<td>3.70 ± 2.27</td>
</tr>
<tr>
<td>INF-γ</td>
<td>3.1 (2.0–4.4)</td>
<td>2.30 (2.0–5.2)</td>
</tr>
<tr>
<td>MDA (nmol/ml)</td>
<td>3.43 ± 2.77</td>
<td>3.58 ± 3.61</td>
</tr>
<tr>
<td>GPX (mU/mg)</td>
<td>2.74 (1.59–5.34)</td>
<td>2.47 (1.45–3.86)</td>
</tr>
<tr>
<td>SOD (IU/mg)</td>
<td>12.50 ± 6.37</td>
<td>14.69 ± 7.80</td>
</tr>
<tr>
<td>Viral load</td>
<td>1.60 ± 1.65</td>
<td>1.79 ± 4.10</td>
</tr>
</tbody>
</table>

*HIV positive versus HIV negative.*
spectrophotometric detection of TBARS overestimates the results. More accurate and improved detection methods have yielded healthy plasma MDA values in the range of 0.1–1 nmol/ml (Karatas et al., 2002; Del Rio et al., 2005). The slightly higher mean MDA values of our controls (1.24 ± 0.69 nmol/ml) may reflect the commented overestimation, but they are clearly lower than the results observed among patients affected by HCV infection.

Nonalcoholics showed higher GPX activity compared to drinkers infected with HCV. In fact, only alcoholics with HCV infection showed significantly reduced GPX activity compared to controls. Antioxidant enzymes synthesis is enhanced during infection showed significantly reduced GPX activity compared to alcoholics infected with HCV. In fact, only alcoholics with HCV infection. In fact, only alcoholics with HCV infection. 

The reasons for such discrepancies, as well as for those regarding cytokines’ results, are unclear, although it is important to keep in mind that in some of these studies, not in others, only individuals infected by genotype 1 virus were considered, and perhaps the timing of blood extraction also differed among the different studies. Oxidative stress and cytokine secretion are closely related to each other’s inflammatory conditions, such as infection, neoplasia, or any situation leading to an acute organic stress, trigger a cytokine response, and enhance oxidative stress. Cytokines’ half-life is usually short, so the altered levels may be influenced by any acute process. In this sense we believe that the fact that cytokines were determined in our patients at a moment of the programmed biopsy strengthens the validity of the results obtained. On the other hand, estimation of lipid peroxidation merely by TBARS concentration may be considered a shortcoming of our study. In addition, although the number of patients included in the study is enough to fulfill the main objective of this study, i.e., to compare cytokines, MDA, and antioxidants between alcoholics and nonalcoholics, it is too short to analyze differences between the different subgroups in which we can divide our sample according to HIV status, gender, and genotype.

Thus, our results show that cytokine secretion is altered in HCV infection. This alteration mainly consists of a stimulation of Th1 cytokines and an inhibition—or at least, no stimulation—of Th2 cytokines; these changes are especially marked among alcoholics with HCV infection, and are accompanied by raised TGFB. We also found increased MDA levels and decreased GPX activity—especially in alcoholics, supporting the view that oxidative damage plays a role in chronic HCV infection. However, the relationship between MDA and antioxidant enzymes’ activity, cytokine secretion, and liver function alterations are relatively poor.

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