Redox alteration in patients infected by different HCV genotypes

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Chronic hepatitis C virus (HCV) infection plays a pivotal role in hepatocarcinogenesis and has been associated with oxidative DNA damage. Few data have been reported on the general redox state in patients infected with different HCV genotypes. Total antioxidant capacity (TAC) and hydrogen peroxide levels as well as oxidative stress index were measured in serum of hepatitis C chronic patients in relation to genotype, viral load, transaminases level and degree of fibrosis.

Serum was obtained from two-hundred-fifty-two HCV infected patients and twenty-five healthy donors. TAC was measured by TAC Colorimetric Assay and hydrogen peroxide concentration by Hydrogen Peroxide Colorimetric Assay Kit. In HCV infected patients, mean serum TAC was 5.62 mM Trolox equivalents which was significantly lower (p<0.0001) than control group (7.25 mM Trolox equivalents). TAC reduction was particularly evident in patients infected by genotype 2 compared to those infected by genotypes 1, 3 and 4. In parallel, high levels of hydrogen peroxide were found in the serum of infected patients, p=0.0015. Although no statistically significant correlation was found with the degree of fibrosis, transaminases level or viral load, oxidative stress index was higher in HCV infected patients compared to uninfected controls, p=0.003.

The results indicate an imbalance of the redox state in HCV infected patients, with a strong reduction of the total antioxidant capacity and high oxidative stress index. Because oxidative burden may favour disease progression, a novel strategy aimed at counteracting it by using antioxidant molecules as adjunct therapy might represent a useful tool in the management of HCV chronic infection.

Keywords: HCV; redox state; oxidative stress index; hepatocarcinogenesis; virus.

INTRODUCTION

In 1989 the parenterally transmitted agent of non-A, non-B viral hepatitis was identified from a cDNA library, clone 5-1-1, and shown to be derived from a new flavi-like virus termed hepatitis C virus (HCV) [1]. HCV causes both acute and chronic infection. Acute HCV infection is usually asymptomatic and only rarely is associated with a life-threatening condition. Actually, about 15-45% of the infected persons clear the virus spontaneously within 6 months without any treatment. The remaining 55-85% of persons will develop chronic HCV infection, which is a major public health issue that continues to cause annually around 700,000 deaths due to hepatitis C-related liver diseases. Among chronic-
ic patients, the risk of developing liver cirrhosis is between 15-30% within 20 years (WHO Fact sheet 2016) [2].

HCV is an enveloped RNA virus classified in the genus *Hepacivirus* in the *Flaviviridae* family. The genome is a positive sense, single stranded RNA molecule approximately 9.6 Kb in length that encodes a single large polyprotein that will be co- and post-translationally processed into 3 structural proteins (core, E1 and E2) and 7 non-structural proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [3].

There are 7 major HCV genotypes which are divided into 67 subtypes [4]. Genotype 1 is the most prevalent worldwide, comprising 83.4 million cases (46.2% of all HCV cases), approximately one-third of which are in East Asia. Genotype 3 is the next most prevalent globally (54.3 million cases, 30.1%); genotypes 2, 4, and 6 are responsible for 22.8% of all cases; genotype 5 comprises the remaining <1%. While genotypes 1 and 3 dominate in most countries irrespective of economic status, the largest proportions of genotypes 4 and 5 are in lower-income countries [5]. Genotype 7 was isolated in Canada from a Central African immigrant [6]. In Italy the most prevalent genotype is HCV 1b [7]. Correct HCV genotyping is essential to predict prognosis and treatment duration [8].

There is accumulating evidence that HCV induces oxidative stress that in turn affects virus replication as well as progression and severity of HCV infection [9]. However, the effect of oxidation on HCV replication is not univocal. Some studies reported a positive effect of oxidative stress on virus replication, while others showed that oxidative stress suppressed viral replication [10, 11].

In the current study, the total antioxidant capacity (TAC) and serum levels of hydrogen peroxide were investigated in hepatitis C chronic patients infected by different HCV genotypes. The redox state was also evaluated in relation to the level of ALT, viral load and degree of fibrosis.

### PATIENTS AND METHODS

The study was carried out on outpatients of the University Hospital Tor Vergata, Rome, Italy. It included two hundred-fifty-two adult patients infected with HCV-genotypes 1a/b (N=106), 2 (N=58), 3 (N=53), 4 (N=35) and twenty-five blood donors as group control. The clinical features and laboratory findings of the patients enrolled in the study are reported in Table 1.

The genotypes were determined using the Abbott RealTime HCV Genotype II assay [12]. Serum of patients and control group was separated using a benchtop centrifuge and stored at -80°C until analysis of the oxidative status. The study was carried out on residual samples otherwise destroyed obtained during routine virological testing. The clinical information was handled in an anonymous way transforming the admission code in an alphanumerical code to keep anonymous the identity of the patient. Because of the retrospective nature of the study, no informed consent is required.

### Total Antioxidant Capacity

Serum Total antioxidant capacity was measured using a Total Antioxidant Capacity (TAC) Colorimetric Assay kit (BioVision Incorporated, Milpitas, CA, USA). The kit can measure either the combination of both small molecule antioxidants and proteins or small molecules alone in the presence of the proprietary Protein Mask. Cu2+ ion is converted to Cu+ by both small molecule and protein. The Protein Mask prevents Cu2+ re-

| Table 1 - Demographics and laboratory findings of HCV positive patients examined. |
|------------------------------------------|------|------|
| **Sex:** | **Male** | **Female** |
| Age | 57.3 ± 13.1 |
| **Transaminase:** | | |
| ALT (U/L) | 89.4±105.1 |
| AST (U/L) | 67.8±72.4 |
| **HCV Genotype** | **Freq.** | **Percent** |
| 1 | 106 | 38.69 |
| 2 | 58 | 21.17 |
| 3 | 52 | 18.98 |
| 4 | 33 | 12.04 |
| HCV- | 25 | 9.12 |
| Total | 274 | 100 |
| **Liver damage:** | | |
| Fibrosis | 46 |
| Cirrhosis | 69 |
duction by protein, enabling the analysis of only the small antioxidant molecules. The reduced Cu+ ion is chelated with a colorimetric probe giving a broad absorbance peak around 570 nm, proportional to the total antioxidant capacity. Trolox is used to standardize antioxidants, with all other antioxidants being measured in Trolox equivalents.

**Hydrogen Peroxide (H₂O₂)**
The quantification of H₂O₂ in the serum patients was performed using Hydrogen Peroxide Colorimetric/Fluorometric Assay Kit (BioVision Incorporated, Milpitas, CA, USA). BioVision’s Hydrogen Peroxide Assay Kit is a highly sensitive, simple, direct and HTS-ready colorimetric and fluorometric assay for measuring H₂O₂ in biological samples. In the presence of Horse Radish Peroxidase (HRP), the OxiRed Probe reacts with H₂O₂ to produce product with color (λ_{max} = 570 nm) and red-fluorescent (Ex/Em=535/587 nm) [13].

**Oxidative stress index**
Oxidative stress occurs when there is an imbalance between reactive oxygen species production and antioxidant defence activity. Oxidative stress index is an indicator of the degree of the oxidative stress, and is given by the ratio of total peroxide to the total anti-oxidant potential [14].

**Statistical analysis**
Differences in levels of serum TCA and H₂O₂ means between infected patients and controls were assessed using Student’s t-test. Furthermore, One-Way ANOVA and ANOVA with Tukey’s post hoc multiple comparisons of the means were used to test differences between the four genotypes groups (1a/b, 2, 3, and 4) and controls. Results were reported as means ±SD. A P-value <0.05 was considered statistically significant. All analyses were performed using the R software/environment (version 3.2.2).

**RESULTS**
In the patient and control groups, the mean ± SD of TAC in the serum varied in relation to the infecting HCV genotypes. The lowest level of TAC was measured in patients infected by genotype 2. Genotypes 3 and 4 showed similar level, while genotypes 1a and 1b had the highest level of TAC. An increase in the level of H₂O₂ was observed in all four genotypes investigated. Data are summarized in Table 2.

Patients infected with genotype 2 or genotype 4 presented levels of ALT lower than patients infected with genotype 1a/b or 3, but this association was not statistically significant, Table 2. Furthermore, no statistically significant association was observed between the levels of TAC and H₂O₂ and those of ALT and viral load. Similarly, it was not found a significant correlation between the levels of TAC and H₂O₂ and the degree of fibrosis or cirrhosis in those patients where this information was available (115 patients). It cannot be excluded that sample size affected this result.
The oxidative stress index was higher in HCV infected patients compared to uninfected controls, and this association was statistically significant, \( p=0.0030 \). When this association was evaluated in relation to the single genotypes, it remained significant for genotypes 1 (\( p=0.0024 \)), 2 (\( p=0.0019 \)) and 3 (\( p=0.0142 \)), while a trend of association was observed for genotype 4 (Table 2).

### DISCUSSION

DNA damage caused by reactive oxygen species (ROS), UV, environmental mutagens and background radiation can lead to genomic instability and cancer, unless DNA damage is recognized and repaired. There is accumulating evidence that both HBV and HCV chronic infection have an important role in the generation of oxidative damage, which has a pivotal role in hepatocarcinogenesis [15]. Increased oxidative stress is a hallmark of HCV chronic infection [10, 16, 17]. Elevated levels of reactive aldehydes (malondialdehyde and 4-hydroxy-2-nonenal) produced by lipid peroxidation have been measured in HCV patients in the serum, peripheral blood mononuclear cells and liver as well as elevated levels of 8-hydroxydeoxyguanosine in liver [18-20]. Recently, the role of different HCV genotypes in the generation of oxidative stress has been investigated by Ansari et al. [21]. They observed a decrease in the level of total antioxidant capacity in HCV infected patients compared to the control group. Considering the different genotypes, the lowest level of antioxidant capacity was observed in patients infected with genotypes 1a/1b, followed by genotypes 4, 2a/2c, 2b and 3a, respectively [21].

In our study, the level of TAC was lower in genotype 2 followed by genotypes 3 and 4 and 1a/1b. Despite these differences at genotype level, the overall TAC level was significantly reduced in all patients in line with the previous observation [21]. Also, the oxidative marker hydrogen peroxide was high in all infected samples compared to the control, \( p=0.0015 \). Accordingly, the oxidative stress index was found significantly increased in all infected patients compared to the control. When this association was evaluated in relation to the specific genotype, a significant association was found for genotypes 1, 2 and 3, and a trend of association for genotype 4. The small sample size of genotype 4 might explain this result.

No correlation was found between viral load, degree of fibrosis or ALT level and oxidative stress. Decreased TAC and increased \( \text{H}_2\text{O}_2 \) and oxidative stress index were reported also in a study by Bolukbas et al., who examined hepatitis B chronic patients affected by cirrhosis [14]. This clinical condition along with poor nutrition status diminishes the ability of the enzymatic antioxidant systems to reduce oxidative compounds favoring oxidative damage. In the case of chronic hepatitis C, a consistent body of literature demonstrated that chronic HCV infection correlates with increased incidence of oxidative DNA damage [14, 22]. HCV induces production of reactive oxygen species not only through inflammation but also in absence of it through its proteins. Actually, HCV core, NS3 and NS5A proteins have been linked to oxidative stress [22-26]. So, eradication of HCV infection by effective antiviral therapy is key to remove this noxious stimulus on liver cells and abolish the production of ROS induced by the virus. New directing-antiviral agents (DAA) have emerged as very promising therapeutic approach with an efficacy close to 100%, minimal side effects and short duration treatment [27].

<table>
<thead>
<tr>
<th>HCV Genotypes</th>
<th>HCV-</th>
<th>1a/1b</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>ALT (U/L)</td>
<td>81.42±60.07</td>
<td>65.25±71.33</td>
<td>103.7±81.39</td>
<td>53.14±36</td>
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<tr>
<td>HCV-RNA (IU/ml)</td>
<td>6427000</td>
<td>2022000</td>
<td>1652000</td>
<td>1296000</td>
<td></td>
<td></td>
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<tr>
<td>TAC (nmol/μl)</td>
<td>7.25±2.31</td>
<td>5.62±2.79</td>
<td>4.27±2.11</td>
<td>4.78±2.28</td>
<td>4.78±2.36</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>H2O2 (nmol/μl)</td>
<td>0.024±0.020</td>
<td>0.171±0.521</td>
<td>0.149±0.246</td>
<td>0.172±0.494</td>
<td>0.11±0.099</td>
<td>0.0015</td>
</tr>
<tr>
<td>Oxidative stress index</td>
<td>0.011±0.005</td>
<td>0.046±0.17</td>
<td>0.043±0.023</td>
<td>0.055±0.18</td>
<td>0.053±0.15</td>
<td>0.003</td>
</tr>
</tbody>
</table>
CONCLUSIONS

The results indicate an imbalance of the redox state in HCV chronic patients, with a strong reduction of total antioxidant capacity and high oxidative stress index. Because oxidative stress may favor liver disease progression, a novel strategy aimed at counteracting it by using antioxidant molecules as adjunct therapy might represent a useful tool in the management of chronic HCV infection [28].

Authors contributions

DL, SB and MC designed the study and DL performed the experiments. FS performed statistical analysis. CD collected the sera. MA provides clinical and demographic information. ATP, LN revised critically the manuscript. DL and MC wrote the paper and participated to the critical revision of the manuscript.

Conflict of interest

The authors declare that the research was conducted in absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES


