Nitric Oxide in Patients with Chronic Liver Diseases

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Abstract

Aim. Chronic liver disease (CLD) may be accompanied by portal hypertension (PHT). Nitric oxide (NO) system disturbances seem to play a key role in the pathogenesis of CLD and PHT. In this study we aim to assess if in chronic active hepatitis (CAH) and cirrhosis (CIR), CLD severity and etiology can be correlated with the serum level of NO metabolites.

Method. The study was performed on 92 patients divided according to the diagnosis and Child-Pugh class, and a control group of 10 healthy volunteers. Serum nitrite/nitrate and citrulline levels were measured in order to evaluate NO synthesis.

Results and conclusion. In CLD patients there was an increased NO production. In CIR NO synthesis increased more than in CAH. In CIR patients only nitrite/nitrate concentrations were correlated with citrulline levels. NO metabolites from CAH and CIR patients varied according to disease etiology, namely NO synthesis was more important in HCV-CLD than in alcoholic-CLD and HBV-CLD. In CIR patients, NO metabolites level increased with disease severity.

Key words
Chronic active hepatitis - cirrhosis - nitric oxide - portal hypertension - viral etiology - alcoholic

Introduction

Nitric oxide (NO) is a short-life molecule produced by the enzyme known as the nitric oxide synthase (NOS), in a reaction that converts arginine and oxygen into citrulline and NO. There are three isoforms of the enzyme: neuronal NOS (nNOS, also called NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). It is now known that each of these isoforms may be expressed in a variety of tissues and cell types. The transcriptional regulation and the post-translational regulation of the catalytic activity is distinct for each isoform. Only nNOS and eNOS are reported to be constitutive enzymes (1,2).

Nitric oxide (NO) is an important signaling molecule that acts in many tissues to regulate a wide range of physiological processes. Consequently, abnormal regulation and control of NO synthesis affect a number of important biological processes and are involved in a variety of diseases (1,2). The NO system disturbances appear to play a key role in the pathogenesis of chronic liver diseases (CLD) (2-7).

The main characteristics of CLD are progressive hepatic failure and irreversible changes in hepatocellular metabolism (2). CLD may be accompanied by portal hypertension (PHT). PHT is the result of the interplay of numerous static and dynamic forces: increased resistance in intrahepatic circulation, increased portal blood flow, development of systemic hyperdynamic circulation (3-13).
Due to the altered hemodynamic profile, the changes in the mechanical forces (pressure and flow) control the endothelial and smooth muscle cell signaling and their structure and function (14). In the splanchnic and systemic circulation, vasodilator agents become predominant, thus inducing functional hypovolemia and decreased effective arterial blood volume, in spite of expanded plasma and blood volume and in spite of fluid retention. An increased NO release by NOS3 has been considered to play a pivotal role in the pathogenesis of hyperdynamic circulation of PHT patients. It mediates the decrease in vascular tone and reactivity (6,14-20). Also NO synthetised by NOS2 and/or NOS3 seems to be a pathogenetic factor involved in the clinical complications of PHT: hepatopulmonary syndrome, portal cardiomyopathy, hepatorenal syndrome and hepatic encephalopathy (15,16,21-31). Previous studies mentioned a significant increase of serum NO metabolites only in patients with CIR, mostly in advanced stages (17,32-34).

Given the pathophysiological events in CLD and the NO synthesis abnormalities in splanchnic and systemic circulation, this study has aimed to find if NO metabolites and citrulline serum levels can be correlated with disease severity and etiology in chronic active hepatitis (CAH) and cirrhosis (CIR).

**Material and methods**

**Patients**

The study was performed on 92 patients hospitalized in the 4th Medical Clinic, Cluj Napoca, between 2003-2004, according to international ethical regulations. Patients agreed to be admitted into the study after they had been informed about the requirements and aims of the study.

Inclusion criteria were:
- CLD, chronic active hepatitis (CAH) and cirrhosis (CIR), diagnosed by clinical, biochemical, ultrasound, and/or histological criteria before the study;
- active stage of CLD with persistent elevation of liver enzymes (ASAT, ALAT, GGT, ALP);
- etiology: viral HBV or HCV, or toxic alcohol-induced.

Cirrhosis was staged in a Child-Pugh class by using phosphoric acid, vanadium chloride (VCl₃), sodium nitrite (MERCK). The specimen was mixed and then boiled for 5 min. After cooling, absorbance was read at 530 nm, on a double beam spectrophotometer (CECIL 3021). Dilutions of standard commercial photometric assays for liver enzymes (ASAT, ALAT, GGT, ALP), albumin, bilirubin, prothrombin time/INR (MERCK);
- ELISA standard kits for hepatitis B surface antigen (HBsAg), and antibodies to HCV (anti-HCV) (BIOKIT-Barcelona);
- L-citrulline (SIGMA), thiosemicarbazide (INC), diacetyl monoxime solution-5mg/ml, and (1980). Specimens were deproteinized and the supernatant was collected. A chromogenic solution (5mg thiosemicarbazide/100ml acid-ferric solution) was added to 0.1ml of supernatant. After being kept for 30-45 min at room temperature, absorbance was measured at 540nm using a spectrophotometer (CECIL 3021) (36).

L-citrulline concentration was assayed colorimetrically, as described by Boyde and Rahmatullah (1980). Specimens were deproteinized and the supernatant was collected. A chromogenic solution (5mg thiosemicarbazide in 50ml diacetyl monoxime solution-5mg/ml, and 100ml acid-ferric solution) was added to 0.1ml of supernatant. The specimen was mixed and then boiled for 5 min. After cooling, absorbance was read at 530 nm, on a double beam spectrophotometer (CECIL 3021). Dilutions of standard citrulline were run concomitantly (37).

**Chemicals used:**
- standard commercial photometric assays for liver enzymes (ASAT, ALAT, GGT, ALP), albumin, bilirubin, prothrombin time/INR (MERCK);
- ELISA standard kits for hepatitis B surface antigen (HBsAg), and antibodies to HCV (anti-HCV) (BIOKIT-Barcelona);
- L-citrulline (SIGMA), thiosemicarbazide (INC), diacetyl monoxime (SIGMA), acid-ferric solution (sulphuric acid, phosphoric acid, FeCl₃-MERCK);
- Ethylenediamine dihydrochloride, sulphanilamide, phosphoric acid, vanadium chloride (VCl₃), sodium nitrite (MERCK).

For serum nitrite/nitrate and citrulline levels, blood samples were collected at 8 a.m. Before blood sampling, patients were on a 3-day standard nitrate intake diet (35).

Serum was deproteinized prior to NO metabolite analysis. Nitrite/nitrate levels were measured using the Griess assay as previously described (36). Briefly, 100 μl of VCl₃ were added to 100 μl of sample, immediately followed by the addition of the Griess reagents (50μl sulphamidine + 50μl ethylenediamine dihydrochloride). After being kept for 30-45 min at room temperature, absorbance was measured at 540nm using a spectrophotometer (CECIL 3021) (36).

Serum L-citrulline concentration was assayed colorimetrically, as described by Boyde and Rahmatullah (1980). Specimens were deproteinized and the supernatant was collected. A chromogenic solution (5mg thiosemicarbazide in 50ml diacetyl monoxime solution-5mg/ml, and 100ml acid-ferric solution) was added to 0.1ml of supernatant. The specimen was mixed and then boiled for 5 min. After cooling, absorbance was read at 530 nm, on a double beam spectrophotometer (CECIL 3021). Dilutions of standard citrulline were run concomitantly (37).

**Table I**

<table>
<thead>
<tr>
<th>Group</th>
<th>Diagnosis</th>
<th>No. of patients</th>
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<th>Gender (f/m)</th>
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<td>37-63</td>
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<tr>
<td>II</td>
<td>HBV-CAH</td>
<td>9</td>
<td>40-61</td>
<td>5/4</td>
</tr>
<tr>
<td>III</td>
<td>HCV-CAH</td>
<td>12</td>
<td>35-56</td>
<td>7/5</td>
</tr>
<tr>
<td>IV</td>
<td>alcoholic-CIR</td>
<td>22</td>
<td>40-65</td>
<td>6/16</td>
</tr>
<tr>
<td>V</td>
<td>HBV-CIR</td>
<td>15</td>
<td>40-59</td>
<td>6/9</td>
</tr>
<tr>
<td>VI</td>
<td>HCV-CIR</td>
<td>19</td>
<td>44-70</td>
<td>8/11</td>
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<tr>
<td>VII</td>
<td>Control</td>
<td>10</td>
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**Table II**

<table>
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<tr>
<th>Group</th>
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<tr>
<td>VI-B</td>
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<td>V-C</td>
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<tr>
<td>VI-C</td>
<td>HCV-CIR-C</td>
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</tbody>
</table>
Statistical analysis
The statistical analysis of data was performed with Microsoft Excel Software 10.0. All results are expressed as mean±SEM. Comparisons between the groups were made with the Student t test for unpaired data. Significance was established at a p level < 0.05.

Results
Serum nitrites and nitrates
Nitrite/nitrate levels in all CLD patients were higher (67.7±6.15mmol/L) than those in the control group (18.5±1.7mmol/L) (p<0.001) (Fig.1).

In comparison to the control group, CAH patients had a smaller increase of nitrite/nitrate serum concentration (61.56±7.5mmol/L) (p<0.001). CIR patients had a more significant increase of nitrite/nitrate levels (73.85±4.8mmol/L) compared to the control group (p<0.0001). In CIR patients nitrite/nitrate concentration was also significantly higher than in CAH patients (p<0.01) (Fig.1).

In the CAH group, HCV-CAH patients had the most important increase in the nitrite/nitrate levels (66.9±4.7mmol/L) (p<0.01). In the alcoholic steatohepatitis group nitrite/nitrate levels were: 63.7±8.4mmol/L (p<0.01). HBV-CAH patients had the lowest concentration: 54.1±9.5 mmol/L (p<0.01) (Fig.2).

Among CIR patients, serum nitrite/nitrate levels showed the most important increase in Child C patients (88.16±9.4mmol/L). In Child B patients nitrite/nitrate concentration was 83.3±11mmol/L, and in Child D A patients it was 50.1±6.2 mmol/L (p<0.0001) (Fig.3).

The nitrite/nitrate levels in each etiological group from the same Child class were also different. In Child A the most important increase was found in alcoholic-CIR patients (p<0.0001), followed by the HCV-CIR group (p<0.001). The lowest level was in HBV-CIR patients (p<0.0001) (Fig.4).

In Child-B and C patients, the most important increase of nitrite/nitrate concentrations was found in HCV-CIR patients, followed by those from the alcoholic-CIR group (Figs.5,6).

Among CIR patients, serum nitrite/nitrate levels showed the most important increase in Child C patients (88.16±9.4mmol/L). In Child B patients nitrite/
Nitrite/nitrate concentrations in Child C class CIR patients. Results are expressed as mmol/L in nitrite/nitrate formation. *p<0.01 vs control.

**Serum citrulline**

All CLD patients had an increased serum citrulline concentration (40.12±2.4mmol/L) as compared to the control group (22.2±1.5mmol/L) (p<0.01). In the CIR patients the increase was more significant (48.64±3mmol/L) (p<0.001) than in the CAH patients (31.6±1.9mmol/L) (p<0.05). Citrulline levels in CIR groups were also significantly higher than in CAH patients (p<0.01) (Fig.7).

Citrulline increased significantly – up to 33.6±2.2mmol/L (p<0.05) - in patients with alcoholic steatohepatitis. In patients HBV-CAH patients the citrulline level was 31.5±1.5mmol/L (p<0.05), and in HCV –CAH patients 29.7±2.4mmol/L (p>0.05) (Fig.8).

Among the CIR groups, serum citrulline increased most significantly in Child-C patients (56.63±4.4mmol/L) (p<0.0001), followed by CHILD-B patients (45.5±2.8mmol/L) (p<0.001), and by CHILD-A patients (44.1±4.2mmol/L) (p<0.01).

The serum citrulline in CIR patients differed according to etiology. In Child-A group alcoholic-CIR patients had the highest citrulline level. In Child-B and Child-C groups HCV-CIR patients presented the most important increase, followed by alcoholic-CIR patients, while the lowest increase was found in HBV-CIR patients (Figs.9-11).
Discussions

It has been demonstrated that in advanced stages of cirrhosis, because of the PHT, sheer stress stimulates endothelial cells to express NOS3. That is why some studies have found an increased NOS3 mRNA activity. In some PHT complications, NOS2 synthesis also increases (6,30,38). These mechanisms may explain the rise of NO metabolites in patients with severe CLD (17,32-34). In concordance with other reports, the results of our study show a serum nitrate/nitrite concentration increase in CLD patients.

In CAH patients, nitrite/nitrate levels increased, but not very much. There is a discrepancy between our results and those reported by some other studies: we found no differences between nitrite/nitrate levels of CAH patients and healthy controls (17,34). There are also studies with results similar to ours which found NO metabolite changes in CAH patients (39-42). Previous studies showed that in CAH, NO level can be positively correlated with ALAT levels. That is why the different results of other studies may be related to liver failure severity.

We also showed that nitrite/nitrate levels were different in different CAH etiological groups: they were higher in HCV-CAH patients, and lower in alcohol induced steatohepatitis and HBV-CAH patients. Citrulline levels also increased in CAH patients, but compared with the control group the results were not significant. The highest citrulline level was found in alcoholic steatohepatitis patients. Similarly to previous studies, we could not find a correlation between citrulline and nitrite/nitrate levels in CAH patients (2,17).

Circulating citrulline and nitrite/nitrate levels in CIR patients were significantly higher as compared to controls. Previous studies mentioned only an important increase of nitrite/nitrate concentration in CIR patients, which did not correlate with citrulline levels (17).

In CIR patients, citrulline and nitrite/nitrate could be correlated with the Child-Pugh class. This observation was also mentioned by other studies which showed that in advanced cirrhosis nitrite/nitrate levels can be correlated with disease severity, namely with PHT complications (ascites, oesophageal varices, IL-6 level) (2,17).

Previous studies showed that in viral cirrhosis there was an important increase in serum NO metabolites and that HCV infection induced a higher synthesis of NO compared with the control group the results were not compared with those of viral cirrhosis (42,45). According to our results, citrulline and nitrite/nitrate levels differed according to CIR etiological group. As to the nitrite/nitrate concentrations, the HCV-CIR group had the highest levels, followed by alcoholic and HBV-CIR patients. Citrulline level varied according to the etiology, and the results could be correlated with nitrite/nitrate levels. Modulation of the NO pathway may be a future therapeutic option for circulatory disturbances in severe CLD (10,46).

In conclusion we found that CLD patients had an increased NO production. NO metabolite levels varied according to disease etiology. In CIR, NO synthesis increased more than in CAH. In CIR patients, only nitrite/nitrate concentrations could be correlated with citrulline levels. Based on the results of the present study and of other reports, we suggest the analysis of serum NO concentrations in order to evaluate PHT severity.

References

17. Arkenau HT, Stichtenoth DO, Frolich JC, Manns MP, Boker KH. Elevated nitric oxide levels in patients with chronic liver disease and cirrhosis correlate with disease stage and parameters


