NITROSATIVE STRESS AND APOPTOSIS IN EXPERIMENTAL ALCOHOLIC HEPATITIS. AN IMMUNOHISTOCHEMICAL STUDY

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Abstract
The purpose of this immunohistochemical study was to assess the hepatic expression of inducible nitric oxide synthase (iNOS or NOS2) and nitrotyrosine (NT) in order to evaluate the relationship between nitrosative stress and apoptosis, in experimental alcoholic hepatitis.

Keywords: nitrosative stress, apoptosis, experimental alcoholic hepatitis

Introduction
Precise mechanisms that induce the changes that characterize chronic ethanol consumption are not fully understood.

Chronic ethanol consumption is associated with an increase in reactive oxygen and nitrogen species, indicating that hepatotoxic effects of ethanol may be a consequence of nitrosative and oxidative stress [1].

Oxidative stress, defined as an imbalance between oxidant producing systems and antioxidant mechanisms, which result in excessive formation of reactive oxygen species (ROS), has been implicated in a large number of human diseases [2].

In hepatocytes ROS may play a role in a very large cascade of reactions such as nitric oxide (NO) synthesis and metabolism, caspase activity, DNA fragmentation, etc. [3].

Besides its role as an endothelial relaxation factor, NO acts as a messenger that modulate several processes (thrombosis, neural activity,
nonspecific host defense), being also described as a highly toxic and reactive molecule [4, 5].

NO is formed in a reaction catalyzed by the nitric oxide synthase from L-arginine during the conversion to citrulline [6]. Liver contains different forms of NO-synthase: the neuronal form (nNOS, NOS1) in the peribiliar plexus, the inducible form (iNOS, NOS2) in hepatocytes, cholangiocytes, Kupffer and stelate cells, and endothelial form (eNOS, NOS3) in the endothelial cells [3].

Nitric oxide easily reacts with the superoxide anion, producing the highly reactive peroxynitrite. Peroxynitrite can produce novel products such as nitrotyrosine, nitrotryptophan, and nitrated lipids that serve as important biological markers in vivo [7]. Evaluation of nitrotyrosine (NT) expression, along with that of nitric oxide, could indicate further damage of cells, as well as the degree of inflammation in diseases associated with intense oxidative stress [8].

Apoptosis triggered by various stimuli is propagated, primarily, on two major pathways: extrinsic path initiated by the activation of cell death receptors and intrinsic path that involves the release of proapoptotic factors from mitochondria. The end-point of both the intrinsic and extrinsic pathways is the activation of a wide variety of intracellular proteases (caspases) and endonucleases, which ultimately degrade cellular constituents [9]. The intrinsic pathway is triggered by different signals and as a result, mitochondrial proteins are released in cytoplasm. The release of cytochrome c has an important role in apoptosome formation and caspases cascade activation [10].

Materials and methods

Tissue samples

The study was conducted on Wistar rats distributed in three groups: group I - animals that received daily 0.3 - 0.6 ml ethanol 60%/100g body weight, for 16 weeks; group II - animals that received daily the same dose of ethanol and an ornithine solution 10mg/kg body weight, as a possible hepatic protector; control group - consisting of animals that received saline solution, kept under the same conditions and slaughtered at the same time with animals from other groups.

Liver tissue fragments were prelevated and fixed for 48 h in 4% buffered paraformaldehyde. After that, fragments were processed for paraffin embedding and paraffin blocks obtained were then cut at 3 μm with a microtome. Sections obtained were used for the immunohistochemical study.

Immunohistochemical staining for NOS2, NT and cytochrome c

Immunohistochemistry was performed on sections deparaffinized in
xylene and rehydrated through graded ethanol concentration, proceeded for blocking of endogenous peroxidase with H$_2$O$_2$ 1% in methanol and blocking of non-specific antigenic sites, with the appropriate normal serum. After that, we performed an over night incubation at 4°C in a humid chamber with the following primary antibodies: i) rabbit polyclonal antibody anti-NOS2 (1:50, Santa Cruz Biotechnology Inc.); ii) rabbit polyclonal anti-nitrotyrosine (1:1000, Sigma); iii) rabbit polyclonal antibody anti-cytochrome c (1:50, Santa Cruz Biotechnology Inc.). Immunoreactions were amplified using avidin-biotin-peroxidase complex (Vector Laboratories) and developed with diaminobenzidine hydrochloride/H$_2$O$_2$ (Sigma). Nuclear counterstaining was done with hematoxylin. Finally, sections were mounted with cover clips and photographs were taken with a Nikon Eclipse microscope.

**Evaluation of NOS2, NT and cytochrome c expression**

NOS2, NT and cytochrome c positive immunostaining resulted in the emergence of brownish granules in the cytoplasm or in the nucleus of hepatocytes.

A semiquantitative analysis of immunohistochemical reactions was performed after the examination of five microscopic fields (×40) on each slide, assignment of scores, based on the number of positive cells identified in the fields observed and calculation of the average scoring for each case. The final score was used for statistical analysis.

The score was considered: 0 – for 0-5 positive hepatocytes/field; 1 – for 5-10 positive hepatocytes/field; 2 – for 10-20 positive hepatocytes/field; 3 – for more than 20 positive hepatocytes/field.

**Statistics.** All data are expressed as mean ± standard deviation (SD). Data were analyzed by Student-test and ANOVA analysis. A value of P < 0.05 was taken as statistically significant.

**Results and discussion**

In liver tissues obtained from the animals of the control group we observed few positive hepatocytes for cytochrome c (fig. 1), whereas on the biological samples from the animals treated with alcohol we observed an increased number of positive hepatocytes for cytochrome c, uneven distributed in the liver parenchyma (fig. 2, 3). Microscopic analysis of sections taken from animals treated with ethanol-ornithine revealed a less intense immunoreactivity of hepatocytes than that observed in alcohol treated animals. Hepatocytes showing cytoplasmic positive reaction for cytochrome c were distributed predominantly in clusters (fig. 4).
Analyzing the hepatic expression of NOS2, we observed a weak positivity for NOS2 in the liver of the animals from the control group. Some hepatocytes located around the central vein or the portal space showed positive immunostaining in cytoplasm (fig. 5). Instead, the analysis of samples obtained from the animals treated with ethanol had shown increased NOS2 immunoreactivity in the hepatic lobule (fig. 6). Hepatocytes with an intense cytoplasmic or nuclear positivity for NOS2 were detected in all samples taken from the animals of group I.
Sections obtained from animals of group II displayed a decreased immunoreactivity for NOS2. Positive hepatocytes were no longer distributed in the whole hepatic lobule and showed predisposition for an arrangement in clusters (fig. 7).

These findings allowed as to conclude that ethanol administration exacerbates the hepatic expression of NOS2, suggesting that this is a significant way for nitric oxide synthesis in the liver.

These results are consistent with other studies, which have shown that prolonged exposure to ethanol up-regulates the expression of inducible nitric oxide synthase in the liver, which, through the production of NO, contributes to the development of liver dysfunctions induced by ethanol consumption [11, 12].

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**Figure 5**
NOS2 staining in liver from the control group. Positive immunoreaction in the cytoplasm of some hepatocytes located in the proximity of the portal space (x20)

**Figure 6**
NOS2 staining in liver from the group I. Intense positive immunoreaction in many hepatocytes (x20)

**Figure 7**
NOS2 staining in liver from the group II. Cytoplasmic and nuclear positive reaction in hepatocytes arranged in clusters (x20)

**Figure 8**
NT staining in liver from the control group. Rare hepatocytes with cytoplasmic positive immunoreaction (x20)
Through its interactions with reactive oxygen species, nitric oxide can generate compounds that further promote the nitrosative stress and can be involved in the apoptosis modulation [13].

One of the molecular fingerprints of the action of reactive nitrogen species on biological substrates is the nitration of tyrosine residues of proteins to nitrotyrosine (NT).

In this experiment, the hepatic expression of nitrotyrosine was similar to that of NOS2 in samples obtained from the animals of the three groups. Microscopic analysis of samples obtained from the control group revealed that immunoreaction for NT was predominantly negative in hepatocytes. However, there were some hepatocytes with cytoplasmic positive reaction for NT near the portal area (fig. 8).

Analysis of samples obtained from ethanol treated animals showed instead an intense immunoreactivity for NT in hepatocytes throughout the liver lobule, noting the presence of a large number of hepatocytes with cytoplasmic or nuclear positivity (fig. 9).

In samples from ethanol-ornithine treated animals (fig. 10), we observed that nitrotyrosine expression was decreased compared with ethanol treated group, suggesting that ornithine may have a hepatic protective effect. In the case of ethanol-ornithine administration, the hepatoprotective role of ornithine could be explained perhaps by the fact that ornithine is the precursor for putrescine, spermidine and spermine. These polyamines have important biological roles being involved in cellular processes such as DNA and protein synthesis, cell proliferation and differentiation, and can also act as a scavenger for reactive oxygen species [14].

In order to assess the incidence of apoptosis, a semiquantitative analysis was performed by counting hepatocytes with positive immunoreaction for cytochrome c in cytoplasm, using the method described before.
We observed that cytochrome c expression in hepatocytes was significantly increased (P < 0.05) in liver samples obtained from ethanol treated animals compared with those obtained from ethanol-ornithine treated animals or control group (table I). These findings indicate that ethanol administration modulates the expression of cytochrome c in hepatocytes, suggesting that intrinsic apoptosis may be one of the mechanisms involved in the pathogenesis of liver diseases caused by chronic ethanol consumption.

The results obtained after the semiquantitative analysis of NOS2 and NT (table I) indicate that ethanol administration induces a significant increase (P < 0.05) of these proteins expression in liver samples taken from the animals of group I compared with group II or control group, which suggests the enhancement of nitric oxide synthesis, respectively nitrosative stress after alcohol consumption.

<table>
<thead>
<tr>
<th>Score (mean ±SD)</th>
<th>Group I</th>
<th>Group II</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>1.88 ± 0.44</td>
<td>1.15 ± 0.20</td>
<td>0.68 ± 0.22</td>
</tr>
<tr>
<td>NOS2</td>
<td>2.4 ± 0.43</td>
<td>1.17 ± 0.24</td>
<td>0.24 ± 0.08</td>
</tr>
<tr>
<td>NT</td>
<td>2.02 ± 0.39</td>
<td>0.77 ± 0.29</td>
<td>0.04 ± 0.09</td>
</tr>
</tbody>
</table>

The hypothesis that inducible nitric oxide synthase and nitrotyrosine can be considered also markers of apoptosis was verified by analyzing the correlation between hepatic expression of these proteins and that of cytochrome c, a protein known as an important proapoptotic factor.

Results showed a significant direct correlation (P < 0.05) between the hepatic expression of inducible nitric oxide synthase, nitrotyrosine and that of cytochrome c, allowing the idea that these proteins can be considered apoptotic markers in this experiment (fig. 11, 12).
Conclusions
In summary, the hepatic expression of NOS2 and NT was increased in chronic ethanol consumption, indicating that nitrosative stress may be one of the pathogenic mechanisms involved in alcoholic liver diseases. On the other hand, our data suggest that NOS2 and NT could be also considered markers of apoptosis in experimental alcoholic hepatitis.

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