Inducible nitric oxide synthase expression (iNOS) in chronic viral hepatitis and its correlation with liver fibrosis

DANIELA-ELISE TACHE1)#, CAMELIA ELENA STĂNCIULESCU2)#, ILEANA MONICA BANITĂ3), ŞTEFANA OANA PURCARU1), ANA MARINA ANDREI2), VIOLETA COMĂNESCU4), CATĂLINA GABRIELA PISOSCHI2)

1) Department of Functional Sciences, University of Medicine and Pharmacy of Craiova, Romania
2) Department of Pharmacy, University of Medicine and Pharmacy of Craiova, Romania
3) Department of Morphological Sciences, University of Medicine and Pharmacy of Craiova, Romania
4) Department of Pathology, Emergency County Hospital, Craiova, Romania

#Authors with equal contribution.

Abstract
Background: Nitric oxide (NO) production by the action of the inducible nitric oxide synthase (iNOS or NOS2) is increased in tissues that are stimulated by cytokine and endotoxins. The role of NO in the pathogenesis of chronic viral hepatitis is not fully understood but it seems that its overproduction is responsible for the pathological changes under inflammatory conditions.

Aim: In this paper, we analyzed the correlation between immunohistochemical expression of iNOS and liver fibrosis in chronic viral hepatitis.

Materials and Methods: Liver biopsies from patients diagnosed with chronic viral hepatitis B and C were embedded in paraffin and further used for histological staining and immunohistochemical reactions to detect the expression of iNOS and TGF-β.

Results: In samples with low degree of fibrosis, we observed a discrete positivity for iNOS in periportal hepatocytes and the immunohistochemical reaction for TGF-β was limited to the endothelial cells of liver sinusoids and pro-inflammatory cells from the portal tracts. Positive reaction for TGF-β increased with the degree of liver fibrosis, while the expression of iNOS was enhanced in hepatocytes, as well as in bile ducts and endothelial cells.

Conclusions: Infection with hepatitis B and C viruses induces iNOS expression in hepatocytes, suggesting that NO overproduction might have an important role in progression of chronic viral hepatitis to cirrhosis.

Keywords: chronic hepatitis, liver fibrosis, immunohistochemistry, iNOS, TGF-β.

Introduction
Hepatic inflammation, steatosis and fibrosis are either direct consequences of hepatitis B and C virus infection or indirect damage determined by the subsequent inflammation and oxidative stress [1].

An important mediator of chronic inflammatory processes in liver cells could be the nitric oxide (NO). In the liver, like many other organs, NO has many actions and can be derived from multiple cellular sources, for this reason the exact role of NO in regulating cell or organ function is complex, and experimental evidence often appears to be contradictory [2]. This free radical is produced from L-arginine during the conversion to citrulline in a reaction catalyzed by nitric oxide synthase (NOS) [3]. In the liver were discovered all the three isoforms of this enzyme: neuronal – nNOS, endothelial – eNOS and inducible – iNOS [2].

Many studies revealed that iNOS is overexpressed in liver after viral infection [4–6]. NO is considered to have both hepatoprotective and cytotoxic effects depending on conditions but in large amount this molecule could determine liver damage by increasing inflammation and promoting fibrosis [6].

Hepatic fibrosis is a response of the healing process after injury and it is characterized by an accumulation of extracellular matrix (“scar”), which follows chronic hepatic disease [7]. Several cytokines secreted as a response to cellular injury seem to have a central role in the pathogenesis of hepatic fibrosis. A particularly well studied cytokine is transforming growth factor-β (TGF-β), which is widely regarded as profibrogenic in liver injury [8] and increased by oxidative and nitrosative stress [1]. TGF-β exists in mammals in three isoforms (TGF-β1, -β2 and -β3), which have similar properties and acts as a central mediator of liver fibrosis by activation of hepatic stellate cells and secondary production of extracellular matrix [9, 10].

In this study, we assessed the expression of iNOS and TGF-β1 using immunohistochemistry in chronic viral hepatitis (CVH) in relation with fibrosis stage.

Materials and Methods
Samples
We prospectively analyzed specimens collected by transcutaneous liver biopsy from patients with CVH
(15 infected with hepatitis C virus – HCV and six with hepatitis B virus – HBV) who have never received specific antiviral and immunomodulatory therapy obtained from the Department of Pathology, Emergency County Hospital of Craiova, Romania. The study was approved by the Medical Ethics Committee and patients gave their informed consent.

**Histological analysis**

Liver samples were formalin-fixed and paraffin-embedded, cut at 3–4 µm and carried out by deparaffinization, dehydration and staining for trichromic Masson and Gömöri’s silver impregnation in order to assess fibrosis stage from F0 to F4 using Metavir scoring system: F0 – no fibrosis; F1 – portal fibrosis; F2 – portal fibrosis and rare fibrous septa; F3 – septa without cirrhosis; F4 – cirrhosis.

**Immunohistochemical analysis**

Immunohistochemical reactions were performed on selected sections of each liver specimen processed as mentioned before, after deparaffinization, dehydration and antigen retrieval in citrate buffer. Antigens detection was done with one of the following primary antibodies: polyclonal rabbit anti-human iNOS (Santa Cruz Biotechnology Inc., USA), 1:100 dilution, and monoclonal mouse anti-human TGF-β1 (Santa Cruz Biotechnology Inc., USA), 1:200 dilution, followed by incubation with appropriate secondary antibodies. We used the Avidin–Biotin complex (Vectastain ABC kit, Vector Laboratories Ltd., UK) in order to amplify the reactions and 3,3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Co., Germany) and hydrogen peroxide (Merck KGaA, Germany) to develop them. Nuclear counterstaining was done with Mayer’s Hematoxylin. Finally, slides were registered with a Nikon Eclipse microscope coupled to a digital camera.

Evaluation of immunohistochemical reactions was done by two different observers according to the following: immunohistochemical reactions (brown deposits in labeled structures) were graded on a scale from 1 (negative or diffuse weak signal) to 2 (moderate intensity) and 3 (strong intensity of the signal), involving an evaluation of the mean signal in all microscopic fields from the whole slide. Images were finally processed using the Microsoft Office Picture Manager.

For each antibody tested, we performed a negative control in which the primary antibody was replaced by phosphate buffer saline.

**Results**

Following the histological analysis, we were able to classify the chronic viral hepatitis specimens as F1 (n=4), F2 (n=6), F3 (n=8), F4 (n=3) according to the Metavir scoring system. We observed that in all liver specimens from patients with CVH, immunohistochemistry for iNOS revealed positive reaction. Specimens with minimal portal fibrosis (F1) and moderate fibrosis (F2) revealed a discrete and isolated iNOS positive immunoreaction in the cytoplasm of hepatocytes, mainly in those surrounding areas of portal fibrosis (Figures 1 and 2).

Analysis of selected samples with F3 fibrosis showed an increased iNOS immunohistochemical expression. Hepatocytes surrounding the areas of fibrosis, as well as bile ducts and endothelial cells from the portal spaces, displayed a positive reaction in the cytoplasm (Figure 3). The pattern of iNOS positivity in hepatocytes changes with the stage of liver damage. While for the previous stages of fibrosis, we observed isolated clusters of iNOS positive hepatocytes, in the specimens with advanced stages of liver fibrosis positive hepatocytes were evenly distributed inside the hepatic lobule (Figure 4). In this condition, granular immunostaining was observed mainly in the cytoplasm (Figure 5), but also in some nuclei (Figure 6).

The distribution of iNOS but also that of TGF-β1 exhibited an almost identical pattern in patients with HCV and HBV. As we reported, analysis of the expression of the pro-inflammatory cytokine TGF-β1 reveals that in specimens with low and moderate fibrosis the immunohistochemical staining was restricted to the activated cells lining sinusoids mainly in the perportal areas while in cirrhotic specimens the intensity of the immunoreaction increased many hepatocytes and cells from the portal space expressing TGF-β1 (Figures 7 and 8).
Inducible nitric oxide synthase expression (iNOS) in chronic viral hepatitis and its correlation with liver fibrosis

**Figure 3** – iNOS positive hepatocytes circumscribing an area of portal fibrosis; positive cells in bile ducts (CVH – F3, ×100).

**Figure 4** – iNOS positive reaction in hepatocytes from areas with steatosis (liver cirrhosis, ×100).

**Figure 5** – Intense iNOS positive reaction in the cytoplasm of hepatocytes lining a fibrotic area (liver cirrhosis, ×400).

**Figure 6** – iNOS positive nuclei inside the hepatic lobule (liver cirrhosis, ×400).

**Figure 7** – Positive immunoreaction for TGF-β1 in cells lining liver sinusoids (CVH – F1, ×200).

**Figure 8** – Intense positive reaction for TGF-β1 in hepatocytes and epithelial duct cells (CVH – F4, ×100).

**Discussion**

The process by which production of collagen and development of liver fibrosis are stimulated is very complex.

Alteration of the hepatocellular oxidant-antioxidant balance may contribute to the progression of liver damage in CVH. Reactive oxygen and nitrogen species (ROS/RNS) seems to be involved in the development of fibrosis, but the precise mechanisms by which they influence progression of hepatic fibrosis are still not fully understood.

Nitric oxide (NO), an important nitrogen reactive species, is a free radical with controversial roles which...
can be generated in almost all hepatic cells (hepatocytes, Kupffer, endothelial and hepatic stellate cells) in various conditions by the action of one of the nitric oxide synthases (NOS), which oxidize L-arginine [11, 12]. In liver, as in many other tissues and organs, NO has multiple actions at cellular or molecular level. In normal liver, small quantities of NO are generated by eNOS in order to maintain perfusion in liver sinusoids by influencing vascular tonus or permeability [13]. NO could also regulate leukocytes adhesion to liver sinusoids endothelium and inhibit platelets adhesion and aggregation [2, 14]. Reference [13] is mentioning regarding the expression of iNOS in the normal liver, but during chronic inflammation, iNOS may be stimulated, enhancing NO synthesis [13].

In our study, we noticed a positive immunoreaction for iNOS protein in all the cases regardless of the fibrosis stage but with different distribution and intensity. Hepatocytes positivity was mainly cytoplasmic and increased with the degree of fibrosis. A positive immunoreaction could also be observed in the bile duct epithelium, dilated sinusoids and less in the macrophages. Our results are in accordance with studies reporting the same increased iNOS expression in liver hepatocytes mentioning that they reported also an intense expression in the macrophages [5, 6, 15].

The overexpression of iNOS in liver samples with an advanced degree of fibrosis or cirrhosis suggests that HCV/HBV infection is associated with an increased production of NO. NO could have cytoprotective or cytotoxic activity under these circumstances depending on cell microenvironment. In the liver, NO could have a protective effect per se blocking iNOS, apoptosis and TNFα induced hepatotoxicity after inhibition of protease activity, suggesting that iNOS can be an adaptable response to minimize the inflammation in the early stages [15, 16]. Most biological actions of NO seem to be mediated by its interactions with paramagnetic centers in effector proteins, such as heme- or iron-sulfur centers, but NO• is also known to react rapidly with other targets that carry unpaired electrons, such as ROS, to form higher reactive species of nitrogen [12]. Until present, many RNS derived from NO have been described, the peroxynitrite being the one with the highest biologic activity. Peroxynitrite effects may be good or destructive depending on its concentration and local conditions, as well as cell activation levels and reduced glutathione cellular reserve, which act as a scavenger [12, 17]. Protein nitrosylation could be the mechanism for its antiviral effect, the target of nitrosylation being a viral protease, and leads to the inhibition of viral proteins cleavage and consequently to blockage of viral replication [18].

The direct effects of ROS/RNS on different cytokines important in cell signaling are involved in pathogenic mechanisms in chronic liver disease. In liver, ROS act as second messengers, stimulating cell migration, α-procollagen, TGF-β and inflammatory cytokines mRNAs expression in hepatic stellate cells [19]. In chronic liver injury, a broad spectrum of pro-fibrogenic cytokines and growth factors is released from liver cells and has a central role for the development of liver fibrosis because they allow a cross-talk between ECM-producing cells. TGF-β, the key cytokine involved in fibrosis progression, has controversial biological roles, pro-fibrogenic but also anti-inflammatory and immunosuppressive effects, its aberrant expression being observed in different liver diseases [20–22].

In our studies regarding the expression of TGF-β1 isomorph, liver biopsies characterized by low degree of fibrosis (F1 and F2) displayed TGF-β1 positive cells in peri-sinusoidal areas, while in samples with cirrhosis the degree of positivity increased being observed a great number of hepatocytes and cells from the bile ducts positive for TGF-β1 protein [23, 24]. This finding confirms that the expression of TGF-β1 could be correlated with the degree of liver damage to cirrhosis in CVH. Initially secreted mainly by Kupffer cells, TGF-β1 activates hepatic stellate cells and their transdifferentiation into myofibroblasts, highly proliferative, fibrogenic and contractile cells [25]. Subsequently, activated hepatic stellate cells produce TGF-β1 in large amounts, increasing and perpetuating liver fibrosis [26]. Some studies revealed the fact that ROS play a decisive role in the initial phase of fibrosis by integrating different pro-fibrotic stimuli independently of TGF-β and than induce proliferation of hepatic stellate cells, TGF-β and collagen synthesis [27]. Other studies demonstrated that TGF-β stimulates the subsequent production of ROS in various types of cells while ROS activate TGF-β and mediate many of TGF-β’s fibrogenic effects [28].

In our work, we observed a relative correlation between iNOS and TGF-β1 expression, samples with low fibrosis displayed a faint positivity for iNOS and less TGF-β1 positive cells while in those with advanced fibrosis the expression of both proteins was increased. Our results are in accordance with those obtained by other groups that found also a direct correlation between iNOS expression and severity of disease in CVH [6, 14].

The mechanism by which RNS and TGF-β1 potentiated reciprocally to contribute to the development and progression of liver fibrosis is not yet fully understood. Unlike ROS, which may be considered pro-fibrogenic, in some cases NO and RNS could have an anti-fibrogenic activity, being able to activate some metalloproteinases and cytokines which decreases type I collagen level [29–31], but their protective effect, as this study revealed, is present only in the early stages of the fibrotic response and disappear later when other factors may interfere and act synergistically.

Since a growing body of evidence shows the close relationship between nitrosative stress and expression of TGF-β1, a close knowledge of the phenomenon in perpetuating fibrosis during chronic viral infection may help understanding better the disease and contribute to new arising therapies.

**Conclusions**

During the progression of liver injury in chronic viral hepatitis, a permanent relationship between various elements of the liver can be described. Therefore, the increased expression of iNOS leads to augmentation of nitrosative stress via NO, which will further stimulate the activated hepatic stellate cells with secondary enhancement of liver damage by fibrosis via TGF-β1.
Further studies are required to determine whether iNOS inhibitors could be useful in reducing liver disease severity and improve the benefits of antiviral therapies.

Acknowledgments
This paper received financial support through the Project “Excellence program for multidisciplinary doctoral and postdoctoral research in chronic diseases”, Grant No. POSDRU/159/1.5/S/133377, partially supported by the Sectoral Operational Programme Human Resources Development 2007–2013, financed from the European Social Fund.

References

Corresponding author
Ileana Monica Banita, Professor, MD, PhD, Department of Morphological Sciences, Faculty of Medicine, University of Medicine and Pharmacy of Craiova, 2 Petru Rareș Street, 200349 Craiova, Romania; Phone +40351–443 500, e-mail: monica.banita@yahoo.com

Received: May 5, 2014     Accepted: July 25, 2014