

NITRO-OXIDATIVE STRESS, VEGF AND MMP-9 IN PATIENTS WITH CIRRHOTIC AND NON-CIRRHOTIC PORTAL HYPERTENSION

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Abstract

Background and aims. Nitro-oxidative stress may have pathophysiological consequences. The study aimed to assess the nitro-oxidative stress, the vascular growth factor, and metalloproteinase-9 levels in patients with noncirrhotic and cirrhotic portal hypertension.

Methods. Patients with noncirrhotic portal hypertension (n=50) and cirrhotic portal hypertension (n=50) from the 3rd Medical Clinic in Cluj-Napoca Romania were prospectively enrolled between October 2004 and October 2006. A control group of healthy volunteers (n=50) was also evaluated. Nitro-oxidative stress was assessed by measuring serum concentration of nitrites and nitrate, 3-nitrotyrosine, total oxidative status, total antioxidant reactivity, and oxidative stress index. Serum vascular growth factor and matrix metalloproteinase-9 were also determined.

Results. Serum nitrites and nitrate levels significantly increased in both noncirrhotic (p<0.001) and cirrhotic portal hypertension (p=0.057). 3-nitrotyrosine also increased in noncirrhotic (p=0.001) and cirrhotic portal hypertension patients (p=0.014). Total oxidative status showed a significant increase in noncirrhotic (p<0.001) and in cirrhotic portal hypertension (p<0.001), but total antioxidant reactivity did not change significantly. The oxidative stress index increased in both noncirrhotic (p<0.001) and cirrhotic portal hypertension (p<0.001), as well as the serum vascular growth factor (p=0.005 and p=0.01, respectively). In NCPHT patients serum MMP-9 was significantly lower than in the healthy controls (p=0.03) and CPHT patients (p=0.05).

Conclusion. In patients with noncirrhotic and cirrhotic portal hypertension a significant systemic nitro-oxidative stress was found, correlated with an increase of VEGF. MMP-9 decreased in noncirrhotic portal hypertension.

Keywords: nitro-oxidative stress, vascular endothelial growth factor, matrix metalloproteinase-9, non-cirrhotic portal hypertension, cirrhotic portal hypertension.

Introduction

Portal hypertension (PHT) is a clinical syndrome, defined by a portal venous pressure gradient between the

portal vein and inferior vena cava exceeding 5 mmHg [1]. It is classified anatomically according to the site of resistance to blood flow as prehepatic, hepatic, and post-hepatic. Cirrhotic PHT (CPHT) is associated with an elevated hepatic venous pressure gradient predominantly due to raised sinusoidal resistance, while in the non-cirrhotic

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PHT (NCPHT) hepatic venous pressure gradient is normal or only mildly elevated. Some NCPHT diseases progress without any sign of significant liver dysfunction [2,3]. The diseases leading to NCPH are primarily vascular in nature, and frequently PHT is a late manifestation of the primary disease [3]. After PHT develops, it affects extrahepatic vessels from the splanchnic and systemic circulation, by inducing arterial vasodilatation and collateral vessel formation [4,5]. The increase in blood flow leads to a hyperdynamic splanchnic and systemic circulatory state. The mechanisms underlying this phenomenon are not fully understood [6]. An overproduction of nitric oxide (NO) in the splanchnic and systemic circulation seems to be the most important mechanism. NO is synthesized by nitric oxide synthase (NOS). There are four isoforms of NOS: endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and mitochondrial nitric oxide synthase [7].

Three sources of excessive NO synthesis were identified in PHT. The first source is eNOS induced NO synthesis. An eNOS up-regulation was found in the superior mesenteric arteries endothelium even before the development of the hyperdynamic splanchnic circulation [5]. The second source is iNOS-induced NO synthesis. In cirrhosis, due to the intrahepatic blood flow obstruction, liver LPS detoxification is reduced leading to plasma LPS increase. Resident macrophages from the the splanchnic circulation are activated by this circulating LPS and secrete proinflammatory cytokines, which then stimulate iNOS expression in extrahepatic vasculature [5,8]. The third source is nNOS induced NO synthesis. Perivascular nNOS-containing nerves mediate neurogenic vasodilation by releasing NO. Up-regulation of nNOS has been recently demonstrated in experimental rats PHT as an additional pathway for mesenteric smooth muscle relaxation [7,9].

It has been established that collateral vessels formation is stimulated by angiogenic factors, such as vascular endothelial growth factor (VEGF) and that favours PHT progression [10]. At the same time VEGF stimulates NO synthesis by inducing eNOS protein expression and activity [11]. Other studies also reported that NO-mediated angiogenesis was due to endothelial VEGF, creating a vicious cycle between vasodilatation and angiogenesis [7]. Active matrix metalloproteinases (MMP) are also important mediators of inflammation and angiogenesis [12].

No information was available on the possible correlations between the NO synthesis, VEGF and MMP-9 in NCPHT. Therefore, the present study was undertaken to test the hypothesis that systemic release of NO may induce nitro-oxidative stress that influences VEGF and MMP-9 in NCPHT patients. We also aimed to find out if there were significant differences between NCPHT and CPHT regarding systemic nitro-oxidative stress, VEGF and MMP-9 levels.

Materials and methods

Study population

The present prospective study included 210 consecutive patients with NCPHT (n=105) and CPHT (n=105) admitted to the 3rd Medical Clinic in Cluj-Napoca Romania between October 2004 and October 2006, only 50 having NCPHT and 50 with CPHT. The enrolled patients met the inclusion criteria regarding presence of PHT as demonstrated by evidence of porto-systemic vascular derivations (ultrasound or endoscopy) and/or complete obstruction in the portal venous system (ultrasound and/or CT scan) and/or reversed blood flow in the portal vein (Doppler ultrasound). The inclusion criteria for the CPHT group were the presence of cirrhosis diagnosed by liver biopsy, the macroscopic aspect of the liver during surgical intervention and the ultrasound aspect of the liver. In the NCPHT group liver cirrhosis was excluded using the same criteria [13]. All patients were over 18 years old, none of them had ascites or took any medication known to affect blood flow or vascular tonus. The two PHT groups were also compared to a control group of healthy volunteers (n=50). Written informed consent was obtained from all patients prior to the study. The study protocol was reviewed and approved by the Ethics Committee of University of Medicine and Pharmacy Iuliu Hatieganu Cluj Napoca, Romania.

Laboratory analysis

Chemistry. The 3-nitrotyrosine (3NT) ELISA Kit (KA0445) was purchased from ABNOVA c/o EMBLEM (Heidelberg, Germany), MMP-9 (DMP900) and VEGF (DVE00) ELISA Kits were purchased from R&D Systems Europe (Bucharest, Romania). Sulphanilamide (SULF), N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD), vanadium (III) chloride (VCl_3), methanol, diethylether, xylenol orange [o-cresolsulphonphthalein-3,3-bis (sodium methyliminodiacetate)], ortho dianisidine dihydrochloride (3-3'-dimethoxybenzidine), ferrous ammonium sulphate, hydrogen peroxide (H_2O_2), sulphuric acid, hydrochloride acid, glycerol, trichloroacetic acid (TCA), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Taufkirchen, Germany). Analytical grade chemicals were used exclusively.

Blood sampling. Fasting venous blood samples were taken into tubes and serum was separated after centrifugation at 1500 g for 10 minutes. Samples were stored until analysis at $-80^{\circ}C$ for biochemical tests and at $-20^{\circ}C$ for ELISA tests.

Nitric oxide synthesis evaluation. The Griess reaction was used as an indirect assay to determine the total serum nitrite (NO_2^-) and nitrate (NO_3^-) as an indirect marker of NO production (NO_x) [14]. Serum samples were filtered through 10-kDd filters (Sartorius AG, Goettingen, Germany) and contaminant proteins were precipitated by

extraction with a 3:1 (v:v) solution of methanol/diethyl ether. The sample methanol/diethyl ether ratio was 1:9 (v:v) [15]. In brief, in order to reduce nitrate to nitrite, 100 µL of supernatant was added to 100 µL of 8 mg/mL VCl₃, followed by the addition of the 100 µL Griess reagents (50 µL of SULF 2% and 50 µL of NEDD 0.1%). After 30 min incubation at 37°C, the sample absorbance was read at 540 nm. The nitrite levels were calculated by using sodium nitrite as a standard and expressed as nitrite µmol/L. Serum concentrations of protein-bound 3-nitrotyrosine (3NT) formed by the nitration of tyrosine-containing proteins was measured by commercially available ELISA kit, as a marker of nitro-oxidative stress. The results were expressed as 3NT µmol/L [16].

Oxidative stress evaluation. The total oxidative status (TOS) of the serum was measured using a colorimetric assay [17]. This assay evaluates the oxidation of ferrous ion to ferric ion in the presence of the reactive oxygen species in an acidic medium. The ferric ions are detected by reaction with xylenol orange. Assay results were standardized using hydrogen peroxide (H₂O₂) as the oxidative species, and the assay results are expressed in µmol H₂O₂ Equiv/L. The total antioxidant response (TAR) was measured in serum using a colorimetric assay [18]. In this test the rate of hydroxyl radical production by the Fenton reaction was measured by monitoring the changes in the absorbance of coloured dianisidyl radicals. After addition of a serum sample, the hydroxyl radical-initiated oxidative reactions are inhibited by antioxidant present in the serum sample. Inhibition of dianisidyl oxidation prevents the subsequent colour change, thereby effectively evaluating the total antioxidant capacity of the serum. The test was calibrated using Trolox and results are expressed as mmol Trolox Equiv/L. The oxidative stress index (OSI) was found by calculating the ratio of the TOS to the TAR, according to the formula [19]: OSI (Arbitrary Unit) = TOS (µmol H₂O₂ Equiv/L)/TAR

(mmol Trolox Equiv/L). OSI represents a general indicator of the oxidative stress severity.

VEGF and MMP-9 evaluation. Serum concentrations of VEGF and MMP-9 were measured by commercially available ELISA kits. The results were expressed as VEGF pg/ml and MMP-9 ng/ml.

Statistical analysis

All results were expressed as the mean ± standard deviation (SD) whenever data were normally distributed. Otherwise the median and [Q1; Q3] were reported (Q1=first quartile; Q3=third quartile). Statistical comparisons between groups were performed using the one-way ANOVA tests with post hoc comparison by Tukey test. Pearson's and Spearman's correlation analysis were used to calculate statistical relationships between parameters. Values of P<0.05 were considered to be statistically significant. Analysis was performed using the SPSS 20.0 for Windows.

Results

Demographic and etiologic data

Subjects within the groups were matched regarding age or gender (Table I). Most of the patients included in the present study had prehepatic NCPHT (40% idiopathic portal vein thrombosis, 40% idiopathic portal vein cavernoma, 20% other). In the CPHT group, the etiology of liver cirrhosis was as follows: 30% had HBV, 30% HCV, 30% alcoholic cirrhosis, and 10% other etiologies .

Nitric oxide synthesis evaluation

Total serum NOx was significantly increased in the NCPHT patients (p<0.001) and in the CPHT patients (p=0.057) when compared to the control subjects. Furthermore, in the NCPHT group NO synthesis increased more than in the CPHT group (p=0.013). When evaluated

Table I. Demographic data of the study groups.

	NCPHT	CPHT	CONTROL
Age (years) (mean±SE)	42.6±2.20	49.2±1.83	40.3±1.60
Total number of patients	50	50	50
Gender (M/F)	27/23	24/26	25/25

Values are means ± SD. NCPHT, non-cirrhotic portal hypertension; CPHT, cirrhotic portal hypertension; M, male; F, female.

Table II. Laboratory analysis results of the study groups.

	NCPHT	CPHT	CONTROL
NOx (nitrite µmol/L)	32.26±1.70	28.48±2.70	21.57±1.98
3NT (3NT µmol/L)	361.04±54.95	281.30±18.23	169.95±14.48
TOS (µmol H ₂ O ₂ Equiv./L)	177.29±3.95	128.55±5.67	24.15±2.49
TAR (mmol Trolox Equiv/L)	1.10±0.002	1.11±0.004	1.10±0.002
OSI	16.009±0.36	11.65±0.52	2.17±0.22
VEGF (pg/ml)	620.67±115.88	440.41±109.83	223.85±41.10
MMP-9 (ng/ml)	2.0002±0.40	3.87±0.43	4.32±0.95

Values are means ± SD. NCPHT, non-cirrhotic portal hypertension; CPHT, cirrhotic portal hypertension; NOx, nitrates + nitrites; 3NT, 3 nitrotyrosine; TOS, total oxidative status; TAR, total antioxidant reactivity; OSI, oxidative stress index; VEGF, vascular endothelial growth factor; MMP-9, matrix metalloproteinase 9.

through 3NT, NO induced nitro-oxidative stress was significantly increased in both groups of patients, NCPHT ($p=0.001$) and CPHT ($p=0.014$). Furthermore, in NCPHT there was a higher elevation of 3NT than in CPHT ($p=0.05$) (Table II). 3NT values were correlated with NOx levels in both NCPHT ($r=0.902$) and CPHT patients ($r=0.79$).

Oxidative stress evaluation

Comparing the healthy subjects and the PHT patients it was found that TOS increased significantly in both PHT groups, respectively in NCPHT ($p<0.001$) and CPHT ($p<0.001$). There was a more important increase of TOS in the NCPHT than in CPHT ($p<0.001$) (Table II). NCPHT patients TOS correlated with NOx ($r=0.69$) and 3NT ($r=0.84$) levels. TAR determination did not find any significant changes in NCPHT ($p=0.28$) and CPHT ($p=0.37$) patients (Table II). OSI calculation showed a significant increase of the oxidative stress in all NCPHT ($p<0.001$) and CPHT ($p<0.001$) patients. In NCPHT group there was a more important increase of OSI than in CPHT group ($p<0.001$) (Table II). There was also a significant correlation between OSI and TOS in NCPHT ($r=0.75$) and CPHT ($r=0.64$) patients.

VEGF and MMP-9 evaluation

Serum VEGF was significantly increased in NCPHT ($p=0.005$) and in CPHT ($p=0.01$) groups. The difference between NCPHT and CPHT serum VEGF levels was small ($p=0.041$) (Table II). Moreover, in NCPHT and CPHT patients VEGF was correlated with NOx and 3NT. In NCPHT patients serum MMP-9 was significantly lower than in the healthy controls ($p=0.03$) and CPHT patients ($p=0.05$). CPHT group had a MMP-9 concentration comparable with than the controls ($p=0.67$) (Table II). In the NCPHT group serum MMP-9 concentration was negatively correlated with the OSI level ($r=0.64$).

Discussion

In this study, we focused on the evaluation of the systemic nitro-oxidative stress, MMP-9 and VEGF in NCPHT and CPHT patients. We found that in the NCPHT group there was a more important increase of the nitro-oxidative stress and VEGF synthesis than in CPHT group. MMP-9 was reduced only in the NCPHT group.

In the physiopathology of PHT the initiating step seems to be mesenteric splanchnic vasodilation. Previous studies have shown that splanchnic vasodilation is induced by a complex multifactorial mechanism, which is not completely understood [4,5,20,21]. Until now three mechanisms have been identified: increase in local and systemic vasodilator levels, splanchnic vascular hyporesponsiveness to vasoconstrictors [4] and mesenteric sympathetic atrophy [22]. Among the arterial vasodilator mediators, nitric oxide (NO) has the most important role [23].

Nitrate (NO₃⁻) and nitrite (NO₂⁻) are end products of NO metabolism, that can be used as an indirect measure of the total NO concentration. By means of NOx assessment we found an important increase in NO synthesis in both NCPHT and CPHT patients. Our results were in agreement with previous reports that stated that in PHT hyperactive endothelial cells with increased eNOS activation play an important role in modulating the vascular changes from the splanchnic and systemic circulation [22]. Many factors that are increased in PHT, including shear stress, vasopressin, angiotensin II, and norepinephrine, can increase eNOS activity [24,25]. In PHT bacterial translocation from the gut into mesenteric lymph nodes is an early mechanism increasing TNF α and eNOS [25,26].

Previous studies demonstrated the involvement of iNOS in the hyperdynamic circulation of PHT [5]. In contrast to eNOS, iNOS expression is principally modulated by transcriptional factors. Among these is NF- κ B, which can be activated by different stimuli including LPS, inflammatory cytokines and oxidative stress [7,27]. In PHT intestinal permeability is increased by the increased portal pressure, causing influx of gut flora-derived LPS [5,7]. iNOS produces almost hundred times more NO compared to eNOS [7]. Consistent with the current report was that NO synthesis increased more in NCPHT than in CPHT patients.

The ratio ROS/RNS plays an important physiological role in the cell, by regulating cell signalling, proliferation, differentiation and apoptosis. In the oxidative stress there is an imbalance between the formation of ROS and RSS, and the total antioxidant defending capacity of the cell [21,28]. Oxidative stress plays an important role in the pathogenesis of PHT because the association of overproduction of superoxide anions and NO observed in the model of partial portal vein ligation, peroxynitrite (ONOO⁻) formation occurs [23]. The amino acid tyrosine appears to be a particularly susceptible target for nitration by ONOO⁻, and the formation of free or protein-associated 3NT has received much recent interest as a potential biomarker for the generation of RNS in vivo [7]. Our results regarding 3NT further suggest that in NCPHT and CPHT there is a significant increase of the nitro-oxidative stress. In the present study, NOx, 3NT, TOS and OSI changes were closely correlated. Consequently, the data obtained indicate that in PHT there was concomitant increase of RNS and ROS, due excessive NO synthesis and high TOS formation. An important observation was also that in PHT patients there was no decrease of the TAR.

In PHT angiogenesis and vascular remodeling leads to the formation of portosystemic collateral circulation [22]. However, there is a significant increase in the production of intestinal VEGF with a subsequent increase in the expression of eNOS in the intestinal microcirculation [29]. Thus, increased intestinal VEGF levels may elevate portal hypertension by stimulating eNOS-derived NO production

and by amplifying angiogenesis in the splanchnic circulation.

In experimental cirrhosis it was also found that plasma VEGF levels were significantly increased [30]. NO is also an angiogenic factor in PHT [25]. Consistent with the previous reports our results in NCPHT and CPHT patients showed an important increase serum VEGF levels, with no difference related to the type of PHT. Moreover, VEGF was correlated with NOx and 3NT, sustaining the reciprocal influence between VEGF, NO synthesis and the nitro-oxidative stress.

Other studies showed different results. The differences were explained through the existence of VEGF isoforms and by the observation that in patients with liver disease, blood VEGF concentrations may change depending to the stage and etiology of the disease. Moreover, sample handling may also influence the measurement of blood VEGF concentration [30]. A limitation of the present study was that we considered an average without taking into account the disease etiology or stage.

Matrix remodeling occurs mainly due to the action of MMP [31,32]. These enzymes are secreted into the extracellular space as zymogens that require activation by a variety of stimuli. The active enzymes can, in turn, be inhibited by the family of tissue inhibitors of metalloproteinases (TIMP) [33]. Activated macrophages produce NO and MMP-9 [34]. MMP-9 displays significant direct interstitial collagenase activity on native forms of collagen I and III reducing fibrogenesis [12,35-37]. In PHT it is not known whether the MMP-9+ macrophage subpopulation within the portal and periductal regions is specifically recruited to this location, or whether infiltrating or resident monocytes are stimulated to differentiate and express MMP-9 in the inflamed portal microenvironment [33].

Different levels of NO may have different effects on MMP-9 expression [38]. In chronic liver injury, excessive MMP-9 activity induced by PHT shear stress may have the potential to induce initial liver injury [12].

Consistent with the current reports, we found in the present study in PHT patients a decreased level of serum MMP-9 correlated with the high nitro-oxidative stress. Furthermore, in addition to their ability to degrade extracellular matrix, MMP process several non-matrix substrates such as growth factors and cytokines [33]. Consequently, data obtained indicate that this might be a mechanism that induced a close negative correlation between the changes in the levels of MMP-9 and VEGF in NCPHT patients.

In conclusion, our data suggest that in PHT systemic excessive NO release and associated oxidative stress induce together nitro-oxidative stress and VEGF increase, and these changes were more severe in NCPHT than in CPHT patients. In the NCPHT group the reduction of the

serum MMP-9 level may be a mechanism of higher nitro-oxidative stress and the angiogenetic factor VEGF.

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