Relationship between Tetrahydrobiopterin and Portal Hypertension in Patients with Chronic Liver Disease

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Tetrahydrobiopterin (BH4) is an essential cofactor in NO synthesis by endothelial nitric oxide synthase (eNOS) enzymes. It has been previously suggested that reduced intrahepatic BH4 results in a decrease in intrahepatic NO and contributes to increased hepatic vascular resistance and portal pressure in animal models of cirrhosis. The main aim of the present study was to evaluate the relationship between BH4 and portal hypertension (PHT). One hundred ninety-three consecutive patients with chronic liver disease were included in the study. Liver biopsy, measurement of BH4 and hepatic venous pressure gradient (HVPG) were performed. Hepatic fibrosis was classified using the Laennec fibrosis scoring system. BH4 levels were determined in homogenized liver tissues of patients using a high performance liquid chromatography (HPLC) system. Statistical analysis was performed to evaluate the relationship between BH4 and HVPG, grade of hepatic fibrosis, clinical stage of cirrhosis, Child-Pugh class. A positive relationship between HVPG and hepatic fibrosis grade, clinical stage of cirrhosis and Child-Pugh class was observed. However, the BH4 level showed no significant correlation with HVPG or clinical features of cirrhosis. BH4 concentration in liver tissue has little relation to the severity of portal hypertension in patients with chronic liver disease.

Keywords: Hypertension, Portal; Nitric Oxide; Liver Cirrhosis

INTRODUCTION

Portal hypertension (PHT) is a common clinical consequence of chronic liver disease that is characterized by a pathological increase in portal venous pressure and is associated with significant morbidity and mortality (1-3). Portal pressure can be increased by a rise in portal blood flow, an increase in vascular resistance, or both. In cases of cirrhosis, increased intrahepatic resistance to portal blood flow is the primary factor leading to PHT. Although this increase is largely attributable to the structural changes of the liver architecture caused by the cirrhotic process, there is also a dynamic and reversible component that represents up to 30%-40% of the total increased intrahepatic vascular resistance in cirrhosis (4). Various vasoactive substances contribute to the development of PHT. Among these, nitric oxide (NO) is one of the important mediators that paradoxically regulates the intrahepatic and splanchnic/systemic circulation (5-7). Splanchnic overproduction and intrahepatic deficit of endothelial NO contributes to the hyperdynamic circulation. Impairment of the endothelium-dependent response to vasodilatation, a condition that has been called "endothelial dysfunction", is considered one of the mechanisms that leads to the increased vascular tone of cirrhotic liver (8). Indeed, endothelial dysfunction has been attributed to reduced NO bioavailability and to increased vasoconstrictor cyclooxygenase-1 derived prostanoids (9, 10).

Tetrahydrobiopterin (BH4) is an essential cofactor for the adequate generation of NO by nitric oxide synthase enzymes (11-13). If adequate quantities of BH4 are not present, NOS uncoupling will occur and the production of NO is decreased. Previous studies have shown that there is a deficiency of BH4 in cirrhotic livers, secondary to a reduction in the expression and ac-

tivity of the rate-limiting enzyme in BH4 synthesis, guanosine triphosphate-cyclohydrolase I (GTPCH), which is associated with decreased NOS activity and NO availability (14). In a study of carbon tetrachloride (CCl$_4$)-induced cirrhosis in rats, the exogenous administration of BH4 for 3 days increased liver NOS activity and cyclic guanosine monophosphate (cGMP) levels and significantly reduced portal pressure (15). However, clinical studies of BH4 have not been conducted.

The present study evaluated the relationship between BH4 and PHT, the histological grades of hepatic fibrosis using the Laennec scoring system, the clinical stage of cirrhosis and the grade of varices. In addition, we investigated the relationship between PHT and clinical outcomes.

MATERIALS AND METHODS

Patients
A total of 204 patients were enrolled in this study. All patients were admitted to Wonju Severance Christian Hospital between December 2008 and January 2012 for the evaluation of chronic liver disease and received a liver biopsy. The exclusion criteria were as follows: age younger than 19 or older than 75 yr, shock status requiring a vasopressor, active infection (e.g., spontaneous bacterial peritonitis), acute renal failure of any cause, serum creatinine above 3.0 mg/dL, hepatocellular carcinoma or a history of another primary malignancy within 3 yr, underlying severe cardiac illness, transaminases above ten-fold the upper limit of normal, ongoing antiviral therapy, the use of vasoactive drugs (including beta-blockers, nitrates, diuretics and anti-inflammatory drugs), pregnancy or lactation and medical or psychological conditions that would not permit the subject to complete the study or sign the informed consent. Of the 204 patients, 11 patients were excluded from the study. The reasons for the exclusion were serum creatinine above 3.0 mg/dL (4 patients) and failure to measure BH4 (7 patients). A total of 193 patients (159 men and 34 women, mean age of 51.6 ± 8.9, age range of 29-75 yr) were included in the study.

Liver biopsy and histomorphological analysis
Ultrasound-guided liver biopsies were conducted using a needle biopsy gun (Acecut, TSK laboratory, Tochigi, Japan) with a 16 gauge × 11.5 cm needle and a 15 mm biopsy specimen notch. Five-micrometer-thick sections were prepared and were stained with hematoxylin and eosin and Masson-trichrome. A liver biopsy measuring at least 10 mm in length with a width of ≥ 1.2 mm was required for inclusion in this study. In the fragmented biopsies, the total length was approximated by adding the maximum dimensions of each individual fragment. The biopsies prospectively evaluated the degree of fibrosis and cirrhotic change according to the Laennec fibrosis scoring system (16). The Laennec fibrosis scoring system, a modification of the METAVIR system (17), scores fibrosis on a scale of 7 grades, with 0 indicating no definite fibrosis, 1-minimal fibrosis (no septa or rare thin septum; may have portal expansion or mild sinusoidal fibrosis), 2-mild fibrosis (occasional thin septa), 3-moderate fibrosis (moderate thin septa; up to incomplete cirrhosis), 4A-mild cirrhosis, definite or probable, 4B-moderate cirrhosis (at least 2 broad septa) and 4C-severe cirrhosis (at least 1 very broad septum or many minute nodules). In using the Laennec fibrosis scoring system, the thickness of the predominant type of septa in each specimen was chosen, and the smallest nodule was selected for scoring. Fibrosis was evaluated simultaneously and prospectively by a liver pathologist who was blinded to the clinical data.

Measurements of BH4
The BH4 level was measured using a high-performance liquid chromatography (HPLC) analysis after iodine oxidation in acidic conditions, as reported in a previous study (18). To measure the amount of biopterin, 100 μL of liver homogenate was used for acidic oxidation, followed by the addition of 100 μL of 0.2 N HCl. The homogenate was then incubated in the dark for 1 hr after 10 μL of an acidic iodine solution had been added (0.9% iodine and 1.8% potassium iodine in 0.1 N HCl). The reaction was terminated by the addition of 10 μL of 2% ascorbic acid. The centrifuged sample was then subjected to HPLC analysis. Concentrations were expressed as pM per mg of protein. The BH4 levels were derived by subtracting the biopterin levels under a basic oxidative state (biopterin+dihydrobiopterin [BH2]) from the levels under an acidic oxidative state (i.e., total biopterin = biopterin+BH2+BH4). The separation was performed on a Spherisorb ODS3 C18 (particle size 5 mm; length × internal diameter = 250 × 4.6 of 1 mM GL Sci, Japan) using 10 mM Na$_2$PO$_4$ (pH 6.0) at a flow rate of 1.2 mL per minute. Pterins were detected by their native fluorescence (excitation wave length 350 nm, emission 450 nm) using a Hewlett-Packard model 1046A fluorescence detector.

Measurements of HVPG
After overnight fasting, the hepatic venous pressure gradient (HVPG) was measured. The right hepatic vein was catheterized percutaneously through the femoral vein, and the pressures in both the wedged and the free positions were recorded with a 7-F balloon-tipped catheter (Arrow Deutschland GmbH, Erding, Germany). In the case where a shunt was present in the right hepatic vein, HVPG was measured in the middle hepatic vein. All measurements were performed at least in triplicate, and permanent tracings were obtained on a multichannel recorder (19, 20). HVPG was determined by subtracting the free hepatic venous pressure from the wedged hepatic venous pressure (21). An examiner with 12 yr of experience with HVPG measurements performed all HVPG procedures.

Transient elastography

At the same day of measurement of HVPG, after overnight fasting, all patients underwent abdominal ultrasonography. Immediately following the ultrasonography examination, transient elastography was carried out by 2 experienced operators. The Fibroscan device (Fibroscan®, Echosens, Paris, France) consists of a 3.5 MHz ultrasound transducer M probe mounted on the axis of a vibrator. Mild amplitude and low-frequency vibrations (50 Hz) are transmitted to the liver tissue, inducing an elastic shear wave that propagates through the underlying liver tissue. Transient elastography was carried out on the right lobe of the liver, in the intercostal space with the patient lying in dorsal decubitus with the right arm at maximal abduction, as previously described (22, 23). The operator, assisted by an ultrasonic time-motion image, located a liver portion at least 6 cm thick and free of large vascular structures and the gallbladder. Ten successful measurements were performed on each patient. The success rate was calculated as the ratio of the number of successful measurements over the total number of acquisitions. Only liver stiffness measurements with an interquartile range of ≤ 30% of the median value and a success rate of at least 60% were considered reliable. The results are expressed in kilopascals (kPa) with the median value used to represent the liver stiffness measurement.

Esophagogastroduodenoscopy, imaging studies and laboratory parameters for clinical stage

Esophagogastroduodenoscopy, imaging studies (including ultrasonography and computed tomography) and laboratory tests were performed in all patients to determine the clinical stage of cirrhosis, model for end-stage liver disease (MELD) and the Child-Pugh score. The grades of esophageal varices were classified according to the classification of the Japanese Research Society for Portal Hypertension: F1, straight varices not disappearing with insufflations; F2, enlarged tortuous, occupying less than 1/3 of the lumen; and F3, coil-shaped, occupying more than 1/3 of the lumen. The clinical stage of cirrhosis was established by the presence or absence of varices, ascites, and bleeding, and it was classified into 4 stages as follows: stage 1, no varices; stage 2, varices and no ascites; stage 3, ascites ± varices; and stage 4, bleeding ± ascites. Stages 1 and 2 are compatible with compensated cirrhosis, but stages 3 and 4 are compatible with decompensated cirrhosis (24, 25). Laboratory parameters were obtained in all patients on the day of HVPG measurement and included serum albumin, bilirubin, alanine aminotransferase, aspartate aminotransferase, platelet count, hemoglobin, hematocrit, white blood cell count, International Normalized Ratio (INR), blood urea nitrogen, creatinine, electrolytes and C-reactive protein.

Statistical analysis

Statistical analysis was carried out using the IBM SPSS statistics 21. Categorical variables were expressed as proportions and compared using the chi-square and Fisher's exact tests, as appropriate. All results are expressed as the mean ± standard deviation (SD). Normally or near-normally distributed variables were compared using Student's t-test. Non-normally distributed continuous variables were compared using the Kruskal-Wallis test. Linear regression analyses were conducted using the least squares method. One-way ANOVA was used to analyze the relationships among BH4, HVPG, hepatic fibrosis by Laennec fibrosis scoring system, BH+BH2/BH4 ratio, clinical stage of cirrhosis and Child-Pugh class. P values less than 0.05 were considered statistically significant.

Ethics statement

The study protocol was approved by the institutional review board for human research at the Yonsei University Wonju Severance Christian Hospital, Wonju, Republic of Korea (CR 109021). The nature of the study was explained to all patients, each of whom provided written informed consent before the beginning of the study, in accordance with the principles of the Declaration of Helsinki (revision of Edinburgh, 2000).

RESULTS

General characteristics of the patients

The general characteristics of the patients are summarized in Table 1. The etiology of chronic liver disease was classified as alcohol (n = 136, 70.5%), hepatitis B virus (n = 32, 16.6%), alcohol with hepatitis B virus (n = 10, 5.2%), hepatitis C virus (n = 4, 2.1%) alcohol with hepatitis C virus (n = 1, 0.5%) or cryptogenic cirrhosis (n = 10, 5.2%). The Laennec fibrosis stage was classified as F0 (n = 3, 1.6%), F1 (n = 3, 1.6%), F2 (n = 12, 6.2%), F3 (n = 22, 11.4%), F4A (n = 25, 13.0%), F4B (n = 74, 38.3%) or F4C (n = 54, 28.0%). Cirrhosis was present in 153 of the 193 patients. The etiology of cirrhosis was classified as alcohol (n = 105, 68.6%), hepatitis B virus (n = 27, 17.6%), alcohol with hepatitis B virus (n = 8, 5.2%), hepatitis C virus (n = 3, 2.0%), or cryptogenic cirrhosis (n = 10, 6, 5%). The clinical stage of cirrhosis was classified as stage 1 (n = 39, 25.5%), stage 2 (n = 44, 28.8%), stage 3 (n = 45, 29.4%), or stage 4 (n = 25, 16.3%). The mean Child-Pugh and MELD scores were 6.8 ± 1.7 (range 5-12) and 10.4 ± 3.7 (range 4-22), respectively. The mean HVPG was 14.5 ± 4.9 (range 3-27).

Relationship between BH4 and HVPG

Considering the whole patient population, a negative correlation between HVPG and BH4 was observed. Fig. 1 shows the linear regression analysis between HVPG and BH4 ($r^2 = 0.023$, $P = 0.036$). However, because the coefficient of determinant is 0.023, the linear regression fit is poor. Therefore, BH4 is not a useful predictor for HVPG.

Table 1. General patient characteristics

Parameters	Total (n = 193)	Non-cirrhosis (n = 40)	Cirrhosis (n = 153)	P
Age (yr, range)	51.6 ± 8.9 (29-75)	50.6 ± 10.2 (29-69)	51.8 ± 8.5 (29-75)	0.494
Sex (M:F), No. (%)	159 (82.4) : 34 (17.6)	35 (87.5) : 5 (12.5)	124 (81) : 29 (19)	0.3
Etiology, No. (%)				0.196
Alcohol	136 (70.5)	31 (77.5)	105 (68.6)	
HBV	32 (16.6)	5 (12.5)	27 (17.6)	
Alcohol+HBV	10 (5.2)	2 (5)	8 (5.2)	
HCV	4 (2.1)	1 (2.5)	3 (2)	
Alcohol+HCV	1 (0.5)	1 (2.5)	0 (0)	
Cryptogenic	10 (5.2)	0 (0)	10 (6.5)	
BH4 (mM/mg, range)	0.497 ± 0.298 (0-1.31)	0.512 ± 0.284 (0.077-1.082)	0.493 ± 0.302 (0-1.31)	0.724
Total biopterin (mM/mg, range)	0.594 ± 0.313 (0.071-1.310)	0.608 ± 0.309 (0.095-1.276)	0.59 ± 0.315 (0.071-1.310)	0.752
BH+BH2/BH4 ratio	0.324 ± 0.692 (0-7.623)	0.271 ± 0.504 (0-2.927)	0.338 ± 0.734 (0-7.623)	0.506
Albumin (g/dL)	3.4 ± 0.5	3.6 ± 0.4	3.3 ± 0.5	0.001
AST (U/L)	69.2 ± 44.8	74 ± 47.	68.0 ± 44.2	0.471
ALT (U/L)	39.7 ± 38	47.8 ± 53.6	37.6 ± 32.7	0.133
Total bilirubin (mg/dL)	1.9 ± 2.0	1.5 ± 1.5	2.0 ± 2.1	0.112
Prothrombin time (INR)	1.2 ± 0.2	1.0 ± 0.1	1.2 ± 0.2	< 0.001
Platelet count (× 10³/L)	139 ± 97	238.6 ± 142.3	113 ± 58.4	< 0.001
Laennec fibrosis score, No. (%)				
F0 (No definite fibrosis)	3 (1.6)	3 (7.5)		
F1 (Minimal fibrosis)	3 (1.6)	3 (7.5)		
F2 (Mild fibrosis)	12 (6.2)	12 (30)		
F3 (Moderate fibrosis)	22 (11.4)	22 (55)		
F4A (Cirrhosis, mild)	25 (13)		25 (16.3)	
F4B (moderate cirrhosis)	74 (38.3)		74 (48.4)	
F4C (Severe cirrhosis)	54 (28)		54 (35.3)	
HVPG (mmHg)				< 0.001
HVPG ≥ 10, No. (%)	137 (71)	12 (30)	125 (81.7)	
HVPG ≥ 12, No. (%)	119 (62.7)	6 (15)	101 (73.9)	
LSM (kPa, range)	37.2 ± 21.7 (5.4-75)	21.9 ± 16.3	41.8 ± 21.1 (5.4-75)	< 0.001
Child-Pugh's score			6.8 ± 1.7	
Child-Pugh's grade, No. (%)				
A			83 (54.2)	
B			57 (37.3)	
C			13 (8.5)	
Esophageal varices, No. (%)				
F0			57 (37.3)	
F1			41 (26.8)	
F2			42 (27.5)	
F3			13 (8.5)	
Clinical stage of cirrhosis, No. (%)				
1			39 (25.5)	
2			44 (28.8)	
3			45 (29.4)	
4			25 (16.3)	

Results are expressed as the mean ± SD. BH, biopterin; BH2, dihydrobiopterin; BH4, tetrahydrobiopterin; HBV, hepatitis B virus; HCV, hepatitis C virus; PT, prothrombin time; INR, international normalized ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HVPG, hepatic venous pressure gradient; LSM, liver stiffness measurement; kPa, kilo Pascal.

Relationship between BH4 and the Laennec fibrosis scoring system

Fig. 2 shows the relationship between the Laennec fibrosis scoring system and BH4. The Laennec fibrosis score does not correlate with BH4. The mean BH4 levels in the F0 (n = 3), F1 (n = 3), F2 (n = 12), F3 (n = 22), F4A (n = 25), F4B (n = 74), and F4C (n = 54) stages were 0.621 ± 0.336 (range 0.223-0.826), 0.530 ± 0.313 (range 0.205-0.830), 0.501 ± 0.233 (range 0.095-0.886), 0.500 ± 0.315 (0.077-1.082), 0.569 ± 0.342 (0.077-1.310), 0.484 ± 0.302 (0.047-1.258) and 0.472 ± 0.298 (0-1.162), respectively (*P* = 0.867).

However, although not statistically significant, the mean BH4 levels showed a lower trend with increased hepatic fibrosis progression. In addition, higher hepatic fibrosis progression, a Laennec fibrosis score above 2, was associated with a higher trend in the mean BH+BH2/BH4 ratio. The mean BH+BH2/BH4 ratio in the F0, F1, F2, F3, F4A, F4B, and F4C stages were 0, 0.3 ± 0.52 (0-0.09), 0.312 ± 0.31 (0-0.913), 0.32 ± 0.631 (0-2.927), 0.223 ± 0.518 (0-2.343), 0.332 ± 0.9 (0-7.623), and 0.398 ± 0.545 (0-2.577), respectively (*P* = 0.883).
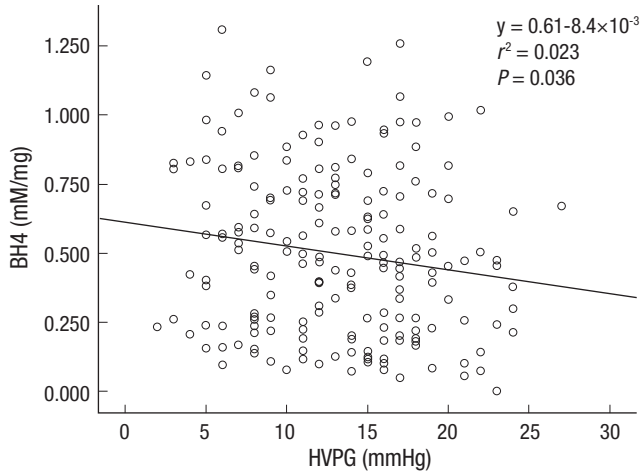
Fig. 1. Linear regression analysis between HVPG (mmHg) and BH4 (pM/mg). There was no significant correlation between HVPG and BH4 ($r^2 = 0.023$, $P = 0.036$). HVPG, hepatic venous pressure gradient; BH4, tetrahydrobiopterin.
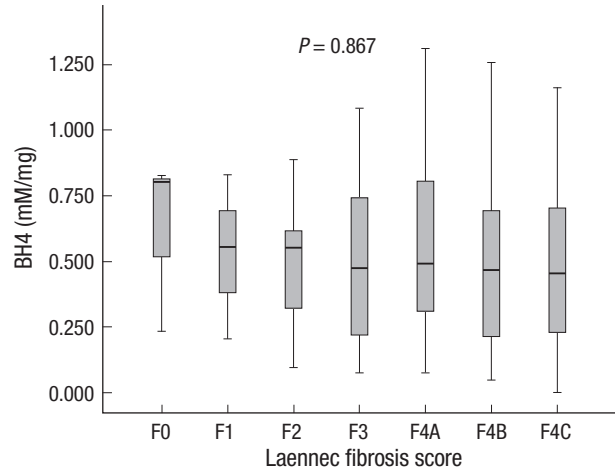

Fig. 2. Correlation between Laennec scoring system and BH4 levels. There was no significant correlation between hepatic fibrosis subclassification according to the Laennec scoring system and BH4 ($P = 0.867$). BH4, tetrahydrobiopterin.
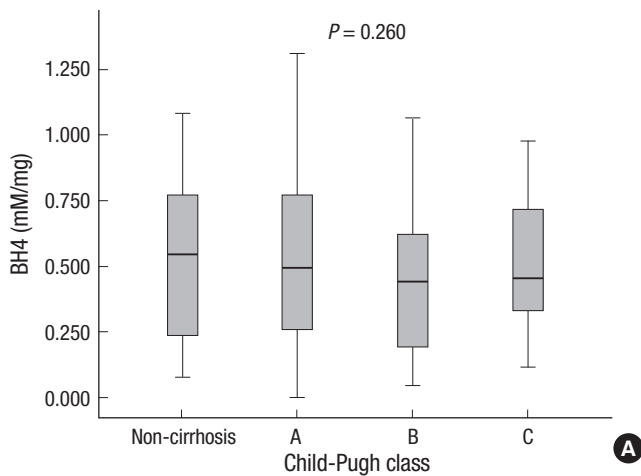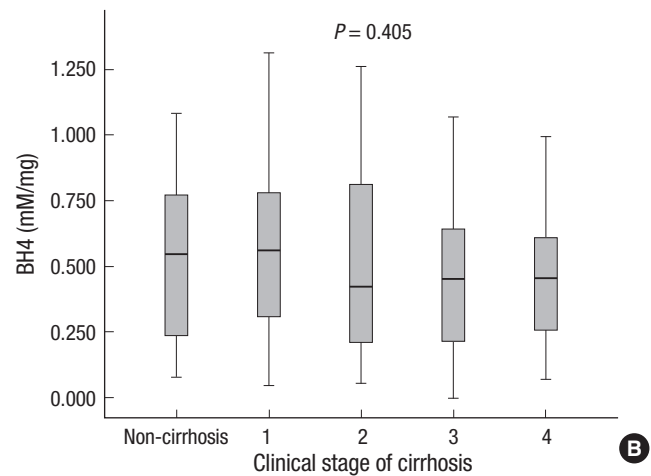

Fig. 3. Relationship between BH4 and clinical outcomes. (**A**) Relationship between Child-Pugh Class and BH4. There was no significant correlation between Child-Pugh Class and BH4 ($P = 0.26$). (**B**) Relationship between clinical stage of cirrhosis and BH4. There was no significant correlation between clinical stage of cirrhosis and BH4 ($P = 0.405$). BH4, tetrahydrobiopterin.

Relationship between BH4 and clinical outcomes

Fig. 3A shows the relationship between BH4 and the Child-Pugh class. The Child-Pugh class does not correlate with BH4. The mean BH4 levels in the non-cirrhosis (n = 40), Child A (n = 83), Child B (n = 58), and Child C (n = 13) classes were 0.512 ± 0.284 (range 0.077-1.082), 0.534 ± 0.320 (range 0-1.31), 0.433 ± 0.278 (range 0.047-1.067), and 0.5 ± 0.298 (range 0.5 ± 0.298), respectively ($P = 0.260$). Fig. 3B shows the relationship between BH4 and the clinical stage of cirrhosis. The clinical stage of cirrhosis does not correlate with BH4. The mean BH4 levels in the non-cirrhosis (n = 40), stage 1 (n = 39), stage 2 (n = 44), stage 3 (n = 45), and stage 4 (n = 25) cases were 0.512 ± 0.284 (range 0.077-1.082), 0.561 ± 0.315 (range 0.047-1.310), 0.509 ± 0.345 (range 0.054-1.258), 0.453 ± 0.272 (range 0-1.067), and 0.435 ± 0.298 (range 0.071-0.994), respectively ($P = 0.405$). The severity of esophageal varices does not correlate with BH4. The mean BH4

levels in the F0 (n = 92), F1 (n = 44), F2 (n = 44), and F3 (n = 13) stages were 0.496 ± 0.290 (range 0-1.310), 0.501 ± 0.312 (range 0.071-1.018), 0.511 ± 0.311 (range 0.054-1.258), and 0.451 ± 0.298 (range 0.071-0.962), respectively ($P = 0.938$). However, although not statistically significant, there was an inverse relationship between the clinical stage of progression of cirrhosis and the mean BH4 levels.

Relationship between HVPG and clinical outcomes

HVPG significantly correlated with the Child-Pugh class. The mean HVPG in the non-cirrhosis (n = 40), Child A (n = 83), Child B (n = 57), and Child C (n = 13) classes were 8.2 ± 3.8 (range 2-17), 12.8 ± 4.9 (range 3-24), 16.4 ± 4.2 (range 8-27), and 17.3 ± 4.3 (range 8-24), respectively ($P < 0.001$). Essentially, as HVPG increased, the Child-Pugh class also increased. HVPG also significantly correlated with the clinical stage of cirrhosis. The mean
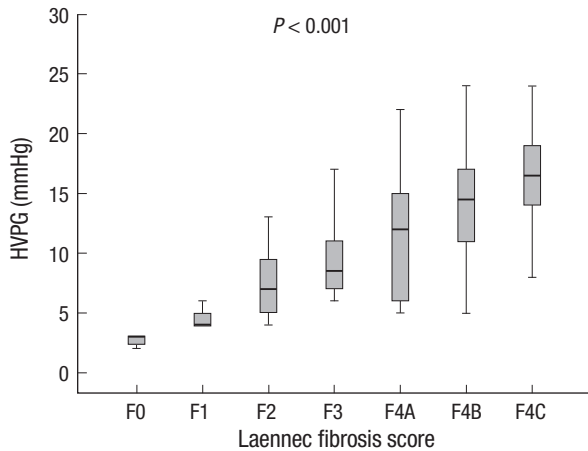
Fig. 4. Relationship between HVPG and Laennec fibrosis scoring system. A statistically significant relationship was observed between HVPG and hepatic fibrosis subclassification using the Laennec fibrosis scoring system ($P < 0.001$). HVPG, hepatic venous pressure gradient.

HVPG in non-cirrhosis (n = 40), stage 1 (n = 39), stage 2 (n = 44), stage 3 (n = 45), and stage 4 (n = 25) cases were 8.2 ± 3.8 (range 2-17), 11.1 ± 3.7 (range 5-19), 14.4 ± 4.9 (range 3-24), 16.8 ± 4.8 (range 5-27), and 15.9 ± 4.0 (range 5-24), respectively ($P < 0.001$). Fig. 4 shows the relationship between HVPG and the Laennec fibrosis scoring system. The mean HVPG in the F0 (n = 3), F1 (n = 3), F2 (n = 12), F3 (n = 22), F4A (n = 25), F4B (n = 74), and F4C (n = 54) cases was 2.7 ± 0.6 (range 2-3), 4.7 ± 1.2 (range 4-6), 8.0 ± 3.8 (range 4-17), 9.6 ± 3.4 (range 6-17), 11.2 ± 5.0 (range 5-22), 14.3 ± 4.4 (range 5-24), and 16.3 ± 4.8 (range 3-27), respectively ($P < 0.001$). A statistically significant, positive correlation between HVPG and liver stiffness was found by linear regression analysis ($r^2 = 0.402$, $P < 0.001$).

DISCUSSION

Intrahepatic vascular resistance is an important component of portal hypertension in chronic liver disease. Endothelial dysfunction in liver sinusoidal endothelial cells decreases the production of vasodilators, such as NO (26). The role of NO in the modulation of intrahepatic vascular resistance has been well demonstrated. eNOS dysfunction in sinusoidal endothelial cells and consequent reduction in NO production or bioavailability plays an crucial role (9, 10). Activation and contraction of hepatic stellate cells also contributes significantly to the dynamic component of intrahepatic vascular resistance. Activated hepatic stellate cells are more susceptible to vasoconstrictor substances than quiescent cells (27). Under condition of reduced NO production and/or impaired NO bioavailability in cirrhosis promotes hepatic stellate cells activation and contraction, leading to sinusoidal remodeling and elevation of the intrahepatic vascular resistance.

NOS are enzymes that catalyze the formation of NO from L-arginine. BH4 is an essential cofactor for endothelial NOS. A previous study using an animal model showed that there is a deficiency of BH4 in cirrhotic livers, secondary to a reduction in the expression and activity of GTPCH (the limiting enzyme in BH4 synthesis), which is associated with decreased NOS activity and NO availability (14). Another in vivo study showed that 3 day supplementation with exogenous BH4 decreased portal pressure in cirrhotic rats (15). However, there has not been any clinical study conducted to evaluate BH4 in human cases. Therefore, we evaluated the relationship between BH4 and HVPG, Laennec fibrosis scoring system, Child-Pugh class and clinical stage of cirrhosis in humans.

In the present study, although HPVG showed a statistically significant correlation with clinical outcomes, including Child-Pugh class, clinical stage of cirrhosis, Laennec fibrosis scoring system and liver stiffness measurement, there is no relationship between BH4 and HVPG. Even though linear regression analysis showed P value smaller than 0.05 for the relationship between BH4 and HVPG, the respective values are widely scattered in the central value as well as very low R square value (0.023), as shown in Fig. 1. It thought to be the statistical limitation that can occur in a large number of subjects. Additionally, BH4 does not correlate with clinical outcomes, including Laennec fibrosis scoring system, Child-Pugh class, clinical stage of cirrhosis and severity of esophageal varices. There is no clear explanation for these results, but several possibilities can be considered.

First, if liver fibrosis progresses past a specific degree, BH4 may not decrease further. In vascular disease, BH4 bioavailability can be limited by superoxide production by various oxidases, forming peroxynitrite and other reactive oxygen species that oxidize BH4 (28). As a result, peroxynitrite and other reactive oxygen species oxidize BH4, via BH2 and BH, which reduces the bioavailability of BH4 and promotes eNOS uncoupling. eNOS then generates superoxide rather than NO, which further reduces NO bioavailability. Through this cycle, if BH4 decreases below a certain level, the progression of hepatic fibrosis will not have an impact on the reduction of BH4.

Second, the ratio of BH4 to oxidized biopterins is as important as absolute BH4 in the determination of eNOS activity. In a study of diabetic mice, endothelial dysfunction was shown to be associated with reduced BH4 levels, but no changes in total biopterins were observed, suggesting that BH4 is oxidized to biologically inactive dihydrobiopterin (BH2) and biopterin (BH) (29). In cirrhosis, the reduced/oxidized pterin ratio (BH4/[BH2+ BH]) has been shown to be markedly reduced in livers from rats with cirrhosis compared with control livers (15). Similarly, although not statistically significant, the present study also showed a direct positive association between hepatic fibrosis progress above a Laennec fibrosis score of 2 and the mean BH+BH2/BH4 ratio.

Third, many substances and drugs interact with BH4. The final step in BH4 biosynthesis is the reduction of 6-pyruvoyltetra-

hydropterin by sepiapterin reductase (SPR). Recently, several studies showed that sulfasalazine, antibacterial sulfa drugs and antidiabetic sulfonylureas are potent inhibitors of SPR and inhibit the biosynthesis of BH4 (30, 31). Additionally, another study has shown that glucocorticoids inhibit the expression of GT-PCH (the rate-limiting enzyme for BH4 synthesis) (32). Metals, including aluminum, cadmium, mercury, lead and manganese, can also affect BH4 biosynthesis by dihydropterin reductase (DHPR) activity. BH4 is mainly regenerated by DHPR (33, 34). In contrast, one study demonstrated that fluvastatin potentiates GTPCH gene expression and BH4 synthesis, thereby increasing NO production and preventing relative shortages of BH4 (35). Additionally, both eplerenone (selective aldosterone antagonist) and enalapril (angiotensin converting enzyme inhibitor, ACEI) reduce NADPH oxidase activity, elevate vascular BH4 levels, and enhance eNOS expression and NO bioavailability (36). Additionally, several studies showed the presence of a chemical interaction between ascorbic acid and BH4 (37, 38). In regard to these data, BH4 concentrations can be affected by sulfa drugs, glucocorticoids, metals, statins, ACEI and vitamin C.

Fourth, in addition to BH4, there are many mechanisms for decreased hepatic NO synthesis in cases of cirrhosis. Increased caveolin expression (cholestasis), decreased Akt-p dependent eNOS phosphorylation, scavenging of NO by superoxide (consequence of reduced superoxide dismutase activity) and increased levels of asymmetric-malondialdehyde (MDA, a serum marker of oxidative stress) all contribute to decreased hepatic NO synthesis in cirrhosis (39).

Finally, in addition to endothelial dysfunction, cyclooxygenase (COX)-derived prostanoids and abnormal angiogenesis could also contribute to increased intrahepatic resistance in portal hypertension. An increased production of a COX-1 derived vasoconstrictor prostanoid has been shown to be responsible for the presence of endothelial dysfunction in liver cirrhosis (10). Additionally, recent studies have demonstrated that angiogenesis plays a critical role in the development and maintenance of splanchnic hyperemia and portosystemic collateralization (40). Multifactorial mechanisms are involved in decreased hepatic NO synthesis in chronic liver disease.

In conclusion, the present study demonstrated that there is no relationship between BH4 and portal hypertension or clinical outcomes in patients with chronic liver disease. BH4 concentrations can be influenced by various substances and drugs. Furthermore, in addition to hepatic endothelial dysfunction with NO, various other factors also affect the development and progression of PHT.

DISCLOSURE

The authors have no conflicts of interest to disclose.

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REFERENCES

1. Suk KT, Baik SK, Yoon JH, Cheong JY, Paik YH, Lee CH, Kim YS, Lee JW, Kim DJ, Cho SW, et al. *Revision and update on clinical practice guideline for liver cirrhosis. Korean J Hepatol 2012; 18: 1-21.*

2. Kim JN, Sohn KM, Kim MY, Suk KT, Jeong SW, Jung HE, Lee SH, Kim SG, Jang JY, Kim YS, et al. *Relationship between the hepatic venous pressure gradient and first variceal hemorrhage in patients with cirrhosis: a multicenter retrospective study in Korea. Clin Mol Hepatol 2012; 18: 391-6.*

3. Kim MY, Baik SK, Suk KT, Yea CJ, Lee IY, Kim JW, Cha SH, Kim YJ, Um SH, Han KH. *Measurement of hepatic venous pressure gradient in liver cirrhosis: relationship with the status of cirrhosis, varices, and ascites in Korea. Korean J Hepatol 2008; 14: 150-8.*

4. Kaneda K, Ekataksin W, Sogawa M, Matsumura A, Cho A, Kawada N. *Endothelin-1-induced vasoconstriction causes a significant increase in portal pressure of rat liver: localized constrictive effect on the distal segment of preterminal portal venules as revealed by light and electron microscopy and serial reconstruction. Hepatology 1998; 27: 735-47.*

5. Wiest R, Groszmann RJ. *The paradox of nitric oxide in cirrhosis and portal hypertension: too much, not enough. Hepatology 2002; 35: 478-91.*

6. Kim MY, Baik SK. *Pathophysiology of portal hypertension, what's new? Korean J Gastroenterol 2010; 56: 129-34.*

7. Kim MY, Baik SK, Lee SS. *Hemodynamic alterations in cirrhosis and portal hypertension. Korean J Hepatol 2010; 16: 347-52.*

8. Gupta TK, Toruner M, Chung MK, Groszmann RJ. *Endothelial dysfunction and decreased production of nitric oxide in the intrahepatic microcirculation of cirrhotic rats. Hepatology 1998; 28: 926-31.*

9. Van De Casteele M, Van Pelt JF, Nevens F, Fevery J, Reichen J. *Low NO bioavailability in CCl4 cirrhotic rat livers might result from low NO synthesis combined with decreased superoxide dismutase activity allowing superoxide-mediated NO breakdown: a comparison of two portal hypertensive rat models with healthy controls. Comp Hepatol 2003; 2: 2.*

10. Graupera M, García-Pagán JC, Abraldes JG, Peralta C, Bragulat M, Corominola H, Bosch J, Rodés J. *Cyclooxygenase-derived products modulate the increased intrahepatic resistance of cirrhotic rat livers. Hepatol-*

ogy 2003; 37: 172-81.

11. Thöny B, Auerbach G, Blau N. *Tetrahydrobiopterin biosynthesis, regeneration and functions. Biochem J 2000; 347: 1-16.*

12. Werner ER, Blau N, Thöny B. *Tetrahydrobiopterin: biochemistry and pathophysiology. Biochem J 2011; 438: 397-414.*

13. Tejero J, Stuehr D. *Tetrahydrobiopterin in nitric oxide synthase. IUBMB Life 2013; 65: 358-65.*

14. Matei V, Rodríguez-Vilarrupla A, Deulofeu R, Colomer D, Fernández M, Bosch J, Garcia-Pagán JC. *The eNOS cofactor tetrahydrobiopterin improves endothelial dysfunction in livers of rats with CCl4 cirrhosis. Hepatology 2006; 44: 44-52.*

15. Matei V, Rodríguez-Vilarrulpla A, Deulofeu R, García-Calderó H, Fernández M, Bosch J, Garcia-Pagán JC. *Three-day tetrahydrobiopterin therapy increases in vivo hepatic NOS activity and reduces portal pressure in CCl4 cirrhotic rats. J Hepatol 2008; 49: 192-7.*

16. Kim MY, Cho MY, Baik SK, Park HJ, Jeon HK, Im CK, Won CS, Kim JW, Kim HS, Kwon SO, et al. *Histological subclassification of cirrhosis using the Laennec fibrosis scoring system correlates with clinical stage and grade of portal hypertension. J Hepatol 2011; 55: 1004-9.*

17. *Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C: the French METAVIR Cooperative Study Group. Hepatology 1994; 20: 15-20.*

18. Lee CK, Han JS, Won KJ, Jung SH, Park HJ, Lee HM, Kim J, Park YS, Kim HJ, Park PJ, et al. *Diminished expression of dihydropteridine reductase is a potent biomarker for hypertensive vessels. Proteomics 2009; 9: 4851-8.*

19. Bosch J, Mastai R, Kravetz D, Navasa M, Rodés J. *Hemodynamic evaluation of the patient with portal hypertension. Semin Liver Dis 1986; 6: 309-17.*

20. Groszmann RJ, Wongcharatrawee S. *The hepatic venous pressure gradient: anything worth doing should be done right. Hepatology 2004: 39: 280-2.*

21. Kim MY, Baik SK, Yea CJ, Lee IY, Kim HJ, Park KW, Kim HK, Suk KT, Kim JW, Kim HS, et al. *Hepatic venous pressure gradient can predict the development of hepatocellular carcinoma and hyponatremia in decompensated alcoholic cirrhosis. Eur J Gastroenterol Hepatol 2009; 21: 1241-6.*

22. Sandrin L, Tanter M, Gennisson JL, Catheline S, Fink M. *Shear elasticity probe for soft tissues with 1-D transient elastography. IEEE Trans Ultrason Ferroelectr Freq Control 2002; 49: 436-46.*

23. Sandrin L, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, Christidis C, Ziol M, Poulet B, Kazemi F, et al. *Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. Ultrasound Med Biol 2003; 29: 1705-13.*

24. De Franchis R. *Evolving consensus in portal hypertension: report of the Baveno IV consensus workshop on methodology of diagnosis and therapy in portal hypertension. J Hepatol 2005; 43: 167-76.*

25. D'Amico G, Garcia-Tsao G, Pagliaro L. *Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. J Hepatol 2006; 44: 217-31.*

26. Kim MY, Baik SK. *Hyperdynamic circulation in patients with liver cirrhosis and portal hypertension. Korean J Gastroenterol 2009; 54: 143-8.*

27. Groszmann RJ. *Hyperdynamic circulation of liver disease 40 years later: pathophysiology and clinical consequences. Hepatology 1994; 20: 1359-63.*

28. Alp NJ, Channon KM. *Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. Arterioscler Thromb Vasc Biol 2004; 24: 413-20.*

29. Alp NJ, Mussa S, Khoo J, Cai S, Guzik T, Jefferson A, Goh N, Rockett KA, Channon KM. *Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. J Clin Invest 2003; 112: 725-35.*

30. Chidley C, Haruki H, Pedersen MG, Muller E, Johnsson K. *A yeast-based screen reveals that sulfasalazine inhibits tetrahydrobiopterin biosynthesis. Nat Chem Biol 2011; 7: 375-83.*

31. Haruki H, Pedersen MG, Gorska KI, Pojer F, Johnsson K. *Tetrahydrobiopterin biosynthesis as an off-target of sulfa drugs. Science 2013; 340: 987-91.*

32. Johns DG, Dorrance AM, Tramontini NL, Webb RC. *Glucocorticoids inhibit tetrahydrobiopterin-dependent endothelial function. Exp Biol Med (Maywood) 2001; 226: 27-31.*

33. Kaufman S. *New tetrahydrobiopterin-dependent systems. Annu Rev Nutr 1993; 13: 261-86.*

34. Nichol CA, Smith GK, Duch DS. *Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. Annu Rev Biochem 1985; 54: 729-64.*

35. Aoki C, Nakano A, Tanaka S, Yanagi K, Ohta S, Jojima T, Kasai K, Takekawa H, Hirata K, Hattori Y. *Fluvastatin upregulates endothelial nitric oxide synthase activity via enhancement of its phosphorylation and expression and via an increase in tetrahydrobiopterin in vascular endothelial cells. Int J Cardiol 2012; 156: 55-61.*

36. Förstermann U, Li H. *Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. Br J Pharmacol 2011; 164: 213-23.*

37. Heller R, Unbehaun A, Schellenberg B, Mayer B, Werner-Felmayer G, Werner ER. *L-ascorbic acid potentiates endothelial nitric oxide synthesis via a chemical stabilization of tetrahydrobiopterin. J Biol Chem 2001; 276: 40-7.*

38. Tóth M, Kukor Z, Valent S. *Chemical stabilization of tetrahydrobiopterin by L-ascorbic acid: contribution to placental endothelial nitric oxide synthase activity. Mol Hum Reprod 2002; 8: 271-80.*

39. Bosch J, Abraldes JG, Fernández M, García-Pagán JC. *Hepatic endothelial dysfunction and abnormal angiogenesis: new targets in the treatment of portal hypertension. J Hepatol 2010; 53: 558-67.*

40. Fernandez M, Vizzutti F, Garcia-Pagan JC, Rodes J, Bosch J. *Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice. Gastroenterology 2004; 126: 886-94.*