Original Article

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Level of Nitric Oxide Metabolite in Liver Cirrhosis Patients with Esophageal Varices

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ABSTRACT

Introduction: Chronic liver disease (CLD) may be accompanied by portal hypertension (PHT). Nitric oxide (NO) disturbance seems to play a key role in the pathogenesis of CLD and PHT. The aim of this study was to clarify the correlation between chronic liver disease stages, esophageal varices presence and nitric oxide disturbance.

Method: The study was conducted on 51 patients divided according to Child-Pugh Classification (CPC) (A = 16, B = 15, C = 10) and a control group of 15 healthy volunteers. All patients included were subjected to full clinical assessment, routine laboratory investigation and upper endoscopy. Serum nitrate and nitrite levels were determined by single step nitrate reductase enzymatic assay and Greiss's reaction colorimetric assay.

Results: Nitrate and nitrite levels in CLD patients were higher (3.02 ± 0.370 and 0.97 ± 0.14 μmol/L) than those in the control group (1.5 ± 0.47 and 0.38 ± 0.01 μmol/L; $p = 0.15$ and $p < 0.001$), respectively. Higher levels of serum nitrite were detected in more advance stages of chronic liver disease (CPC class A 0.38 ± 0.18 μmol/mL, class B 0.64 ± 0.12 μmol/mL, class C 2.8 ± 0.43 μmol/L, $p < 0.01$). No significant difference of nitrate and nitrite levels was found between cirrhosis patients with (2.28 ± 0.42 and 0.6 ± 0.12 μmol/L) and without (2.23 ± 0.46 and 0.53 ± 0.09 μmol/L) esophageal varices ($p = 0.107$ and $p = 0.089$), respectively.

Conclusion: Serum nitrate and nitrite levels can be used as markers of chronic liver disease. The levels of these metabolites increase in advanced liver disease, with no correlation with the presence of esophageal varices.

Key words : Nitric oxide, chronic liver disease, esophageal varices

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INTRODUCTION

Liver cirrhosis represents a late stage of chronic liver disease (CLD) and is characterized by accumulation of fibrosis in the extracellular matrix of the liver. Patients with liver cirrhosis have a shorter survival comparing to normal population. Portal hypertension (PHT) is the major outcome of liver cirrhosis that causes many complications. Esophageal variceal (EV) bleeding is the most serious complication of portal hypertension and most adversely affect patients' survival. Bleeding complication of PHT can be reduced by endoscopy intervention or medication. Development and rupture of EV appear to correlate with the severity of PHT[1].

Nitric oxide (NO) is a potent vasodilator derived from L-arginine by the action of nitric oxide synthase (NOS) enzyme. NO was posted as a key mediator in the pathogenesis of portal hypertension, inducing hyperdynamic circulation and thereby raising portal pressure from increased hepatic blood flow[2]. There are three classes of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelium NOS (eNOS). Hepatic eNOS activity decreases whereas splanchnic and systemic eNOS activity increases in patients with cirrhosis, and this phenomenon appears to correlate with the severity of PHT[3-5]. The half life of NO, however, is too short (a few seconds) to measure directly. NO is degraded in vitro to more stable metabolites, nitrate and nitrite. The levels of these metabolites seem to correlate with progression of PHT.

We performed a cross-sectional analytic study in liver cirrhosis patients to determine the correlation between the level of nitric oxide metabolite and the existence of esophageal varices as well as the severity of liver disease.

PATIENT AND METHODS

This study was conducted at Maharaj Nakorn Chiang Mai Hospital, Thailand, between July 2009 and November 2009. Cirrhotic patients scheduled for esophageal variceal surveillance at the Endoscopy Unit were included in the study and divided into 3 groups according to endoscopic findings; Group 1 (n = 11), cirrhotic patients without esophageal varices: Group 2 (n = 17), patients with small esophageal varices size < 5 mm: Group 3 (n = 13), patients with large esophageal varices size > 5 mm. The control group included 15 healthy volunteers with age and sex-matched. The diagnosis of liver cirrhosis was based on clinical, laboratory and imaging data. Patients with suspected non-cirrhotic portal hypertension (e.g. portal vein thrombosis), intrahepatic malignancy, severe co-morbidity, and patients on non-selective beta-blocker therapy were excluded.

Age, sex, blood pressure, pulse rate, liver function test, INR and complete blood count were measured in all patients. Ten milliliters of venous blood was collected after at least 6-hour fasting and centrifuged at 3,500 rounds/minute for 10 minutes. Serum was removed and stored at -70 ˚C until nitrate and nitrite level measurements were performed.

Nitrite assay

Serum was deproteinized by adding acetonitrile to the serum and centrifuged at 10,000 rounds/minute at 4 ˚C for 10 minutes. Griess's reaction was used for serum nitrite measurement. Briefly, 100 μL of vanadium chloride (VCl_3) was added to 100 μL of the subject sera, followed immediately by Griess' reagents (50 μL of sulphanilamide + 50 μL of ethylenediamine dihydrochloride). Following this, it was kept for 30 minutes at 4 ˚C and absorbance was measured at 540 nm using spectrophotometer. The result was compared with standard sodium nitrite ($NaNO_2$) graph.

Nitrate assay

For nitrate assay, single-step enzymatic assay using Aspergillus nitrate reductase enzyme was used. One-hundred μL of subject sera was mixed with 40 μL of nitrate reductase enzyme (500 U/L) and 10 μL of β-NADH and kept in the dark for 45 minutes. Because β-NADH was oxidized by nitrite, nitrate in serum was reduced to nitrite by nitrate reductase enzyme. The level of β-NADH was the measured by spectrophotometer at 340 nm. The result was compared with standard sodium nitrate ($NaNO_3$) graph.

Statistical analysis

Sample size was calculated to determine significant difference of nitrate or nitrite for esophageal varices for different sizes. From a previous study[6], serum nitrate level was 34.44 ± 3.17 μmol/L in the control group 36.6 ± 7.85 μmol/L is cirrhotics without EV, 51.04 ± 11.5 μmol/L in those with small EV and 109.25 ± 3.54 μmol/L for those with large EV. These results were determined significant by ANOVA test.

$$d = \frac{\delta}{s}$$

$$\delta = \text{MeanMax - MeanMin}$$

$$s = \sqrt{pool\ varience}$$

$$\text{pool variance} = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}$$

$$f = d \times \frac{1}{2} \sqrt{\frac{k + 1}{3(k - 1)}}$$

n_1 = the number of lowest level group
s_1 = standard deviation of lowest level group
n_2 = the number of highest level group
s_2 = standard deviation of highest level group
f = effect size
k = the number of groups

From the above formula, the sample size was calculated at least 11 subjects for each group.

The data were analyzed by SPSS software version 15. The difference of the level of nitrate or nitrite between groups was determined by Krusskal-Wallis test. All results were expressed as mean ± SD. Chi-square analysis was used as a test of significance difference of baseline characters between groups. The result was considered significant if $p < 0.05$.

Results

There was no significant difference in the baseline characteristics among the groups in term of esophageal varices presentation. The major causes of liver cirrhosis were alcohol (n = 12), hepatitis B (n = 9), and hepatitis C (n = 8). (Table 1) The mean serum nitrate level in liver cirrhosis patients was 3.42 ± 0.664 μmol/L, which was significantly higher than in the control group (1.5 ± 0.47 μmol/L), $p = 0.027$. Serum nitrite level in cirrhosis patients was 0.57 ± 0.07 μmol/L, which was also significantly higher than in the control group (0.38 ± 0.12 μmol/L), $p = 0.041$. (Figure 1)

The nitrate and nitrite levels in cirrhosis patients with or without esophageal varices were higher than in the control group, but there was no significant statistical difference ($p = 0.107$ and $p = 0.089$). According to CTP classification, nitrate level in cirrhosis patients CTP class A was 4.17 ± 1.5 μmol/L, in class B was 3.36 ± 0.7 μmol/L and in class C was 2.3 ± 0.48 μmol/L. There was no significant difference of nitrate level between the control group and each of the three CTP classes ($p = 0.155$).

Nitrite level in cirrhosis patients was higher than in the control group and correlated with the severity of

Table 1. Baseline characteristic of cirrhotic patients included in the study.

Characteristic	No EV	Small EV	Large EV	Control	*p*-value
Number	11	17	13	15	
Sex (male/female)	4/7	10/7	6/7	7/8	0.38
Age (year)	53.0 ± 3.6	58.3 ± 2.7	57.3 ± 3.6	55.5 ± 2.4	0.68
BW (kg)	56.0 ± 3.6	58.3 ± 2.7	57.3 ± 3.6	54.8 ± 2.3	0.15
SBP (mmHg)	138.0 ± 6.7	132.0 ± 5.8	125.4 ± 5.3	122.0 ±12.0	0.53
DBP (mmHg)	77.0 ± 3.9	73.6 ± 2.9	67.3 ± 3.5	83.7 ± 3.5	0.08
PR (beats/min)	80.4 ± 4.6	83.6 ± 3.6	85.6 ± 4.0	84.2 ± 3.0	0.83
Cause of cirrhosis				**Total (n)**	
HBV (%)	3 (27.3)	2 (11.8)	4 (30.8)	9	
HCV (%)	4 (36.4)	2 (11.8)	2 (15.4)	8	
Alcohol (%)	1 (9.1)	8 (47.1)	3 (23.1)	12	
HCV+HBV (%)	0 (0)	2 (11.8)	1 (7.7)	3	
Others (%)	2 (18.2)	3 (17.6)	3 (23.1)	8	
CTP grade				**Total (n)**	
A (%)	5 (45.5)	8 (47.1)	3 (23.1)	16	
B (%)	3 (27.3)	5 (29.4)	7 (53.8)	15	
C (%)	3 (27.3)	4 (23.5)	3 (23.1)	10	

mean ± SD

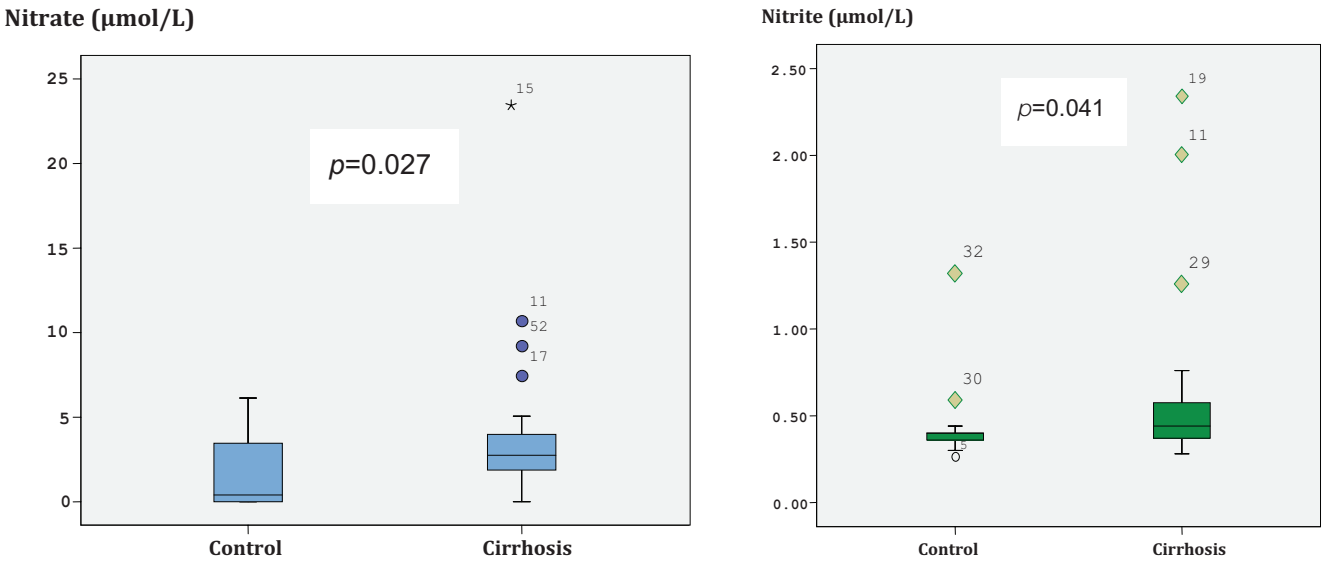

Figure 1. Box-plot of nitrate and nitrite level in cirrhosis and control.

Table 2. Level of NO metabolite in the study.

NO metabolite	No EV	Small EV	Large EV	Control	*p*-value
Nitrate (µmol/L)	2.28 ± 0.42	4.30 ± 1.40	3.10 ± 0.90	1.50 ± 0.47	0.107
Nitrite (µmol/L)	0.53 ± 0.09	0.62 ± 0.12	0.58 ± 0.13	0.38 ± 0.12	0.089
NO metabolite	**CTP A**	**CTP B**	**CTP C**	**Control**	***p*-value**
Nitrate (µmol/L)	4.17 ± 1.50	3.36 ± 0.70	2.30 ± 0.48	1.50 ± 0.48	0.155
Nitrite (µmol/L)	0.39 ± 0.18	0.64 ± 0.12	0.79 ± 0.18	0.38 ± 0.12	<0.001

mean ± SD

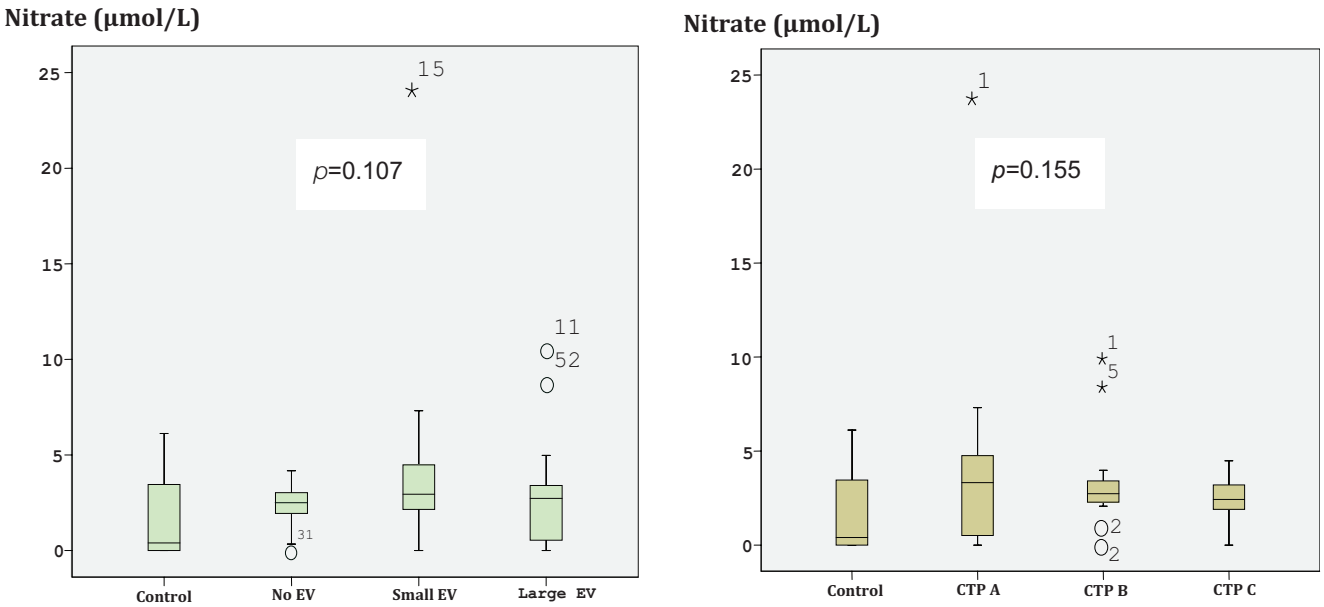

Figure 2. Box-plot of level of serum nitrate in cirrhosis classified by EV size and CTP classes.

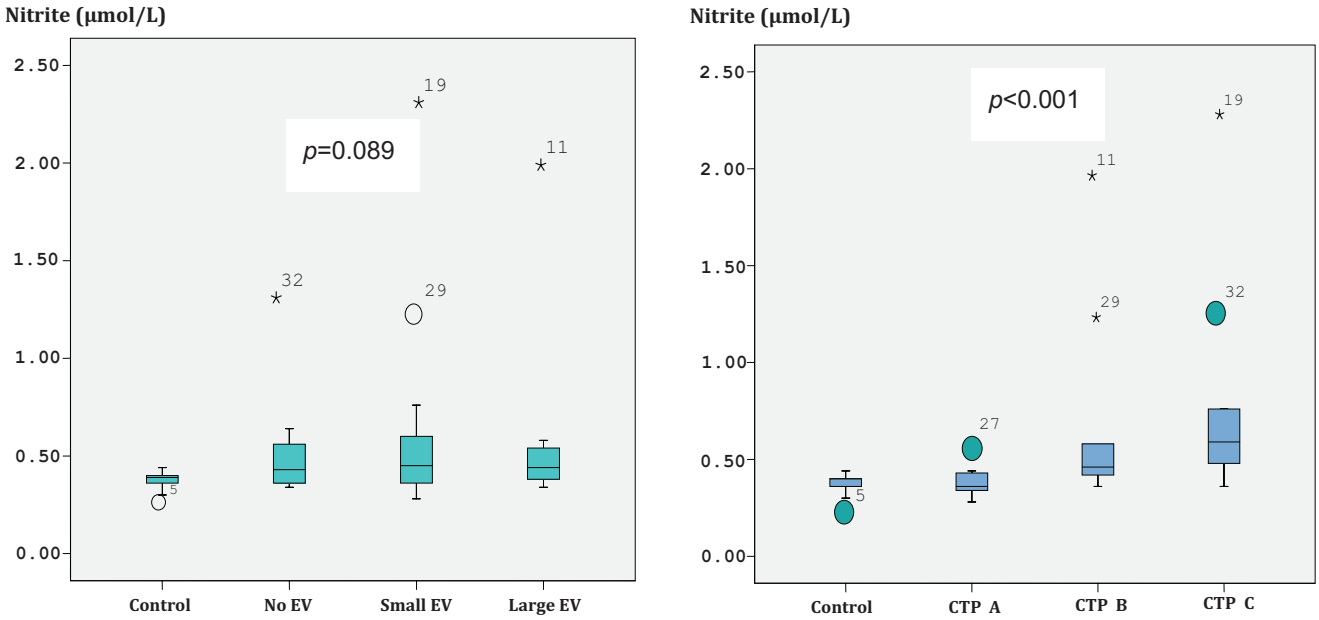

Figure 3. Box-plot of level of serum nitrite in cirrhosis classified by EV size and CTP classes.

liver disease (CTP A = 0.39 ± 0.18 µmol/L, CTP B = 0.64 ± 0.12 µmol/L, CTP C = 0.79 ± 0.18 µmol/L, p <0.001), but there was no significant difference between the control group and CTP A (p = 0.92). Significant difference of nitrate level was observed between the control group and CTP B (p = 0.002), and also CTP C (p <0.001). Nitrite level in CTP C was not significantly higher than in CTP B (p = 0.232). (Table 2, Figure 2 and 3)

Discussion

Our study results showed that the levels of nitrate and nitrite, with of which are metabolites of nitric oxide, significantly increase in patients with liver cirrhosis. These were similar to previous reports[5-7]. However, the levels of nitrate and nitrite in our study appeared to be lower than in other studies. A possible explanation for this observation may be related to the average body weight[8] methods used to measure nitrate and nitrite levels. In our study, and the mean body weight of the control subjects and the study patients was only about 50 kg (average for Asians), much smaller comparing to Caucasians. Regarding measurement of nitrate and nitrite levels, although Griess's reaction was used in most studies including ours, an ultrafilter to filter off protein molecules larger than 10,000 kilodaltons were used in other studies whereas acetonitrile-protein precipitation was employed in ours. Since acetonitrile could precipitate almost all serum proteins with molecular weight above 75 kilodaltons[9], it could lower total nitrate or nitrite level.

The level of nitrate did not significantly rise in severer grades of liver disease as defined by CTP classification and EV size. Serum nitrite level on the other hand, significantly did so in CTP B and CTP C. Such observation differed from a previous study[6] by Assem M. El-Sherif *et al*. in another study by G. ldal Kirkali *et al*., in which significant increase of nitrite level was not noted in more severe liver disease while it was seen with nitrate level. A possible explanation was the different method used for measuring nitrate level i.e. single step enzymatic instead of Greiss's reaction. Another explanation was that the more unstable nitrate could have been degraded before the measurement of nitrate level was completed.

Although nitrite level was significantly higher in more severe liver disease, no significant difference was evident between the control group and the subgroup of early stage cirrhosis or CTP A. In our study, only one patient in the CTP A group had significant ascites reflecting portal hypertension, compared with 12 patients (80%) in CTP B and 10 patients (80%) in CTP C. This discrepancy could be related to different degrees of liver fibrosis and portal hypertension.

Our result suggested that serum nitrate and ni-

trite levels tended to be higher in cirrhotic patients with larger size of EV, as noted in some studies[6,7]. Our data, however, did not reach statistically significant difference with regard to correlation with variceal size. The use of serum nitrate or nitrite serum levels as single or combination markers to predict the size of esophageal varices should be evaluated further in a larger study.

A major limitation in our study was the small number of subjects. Type II error was thus possible in the interpretation of statistical significance. Nitrate and nitrite were not definitely stable. They could disintegrate when stored at room temperature, so that the serum collected had to be transferred immediately in ice-bag for centrifugation and freezing.

CONCLUSION

Nitric oxide metabolites play an important role in the pathogenesis of chronic liver disease. Serum nitrate and serum nitrite levels can be used as markers of liver cirrhosis. The levels of these metabolites appear to increase in advanced liver disease with esophageal varices. Possible correlation of nitrate and nitrite levels with the progression of liver disease and with the occurrence of liver disease complications should be studied further in larger numbers of patients.

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