

Increased Salivary Nitrite and Nitrate Excretion in Rats with Cirrhosis

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Abstract- Increased nitric oxide (NO) formation is mechanistically linked to pathophysiology of the extrahepatic complications of cirrhosis. NO is formed by either enzymatic or non-enzymatic pathways. Enzymatic production is catalyzed by NO synthase (NOS) while entero-salivary circulation of nitrate and nitrite is linked to non-enzymatic formation of NO under acidic pH in the stomach. There is no data on salivary excretion of nitrate and nitrite in cirrhosis. This study was aimed to investigate salivary levels of nitrate and nitrite in a rat model of biliary cirrhosis. Cirrhosis was induced by bile duct ligation (BDL). Four weeks after the operation, submandibular ducts of anesthetized BDL and control rats were cannulated with polyethylene microtube for saliva collection. Assessment of pH, nitrite and nitrate levels was performed in our research. We also investigated NOS expression by real time RT-PCR to estimate eNOS, nNOS and iNOS mRNA levels in the submandibular glands. Salivary pH was significantly lower in BDL rats in comparison to control animals. We also observed a statistically significant increase in salivary levels of nitrite as well as nitrate in BDL rats while there was no elevation in the mRNA expression of nNOS, eNOS, and iNOS in submandibular glands of cirrhotic groups. This indicates that an increased salivary level of nitrite/nitrate is less likely to be linked to increased enzymatic production of NO in the salivary epithelium. It appears that nitrate/nitrite can be transported from the blood stream by submandibular glands and excreted into saliva as entero-salivary circulation, and this mechanism may have been exaggerated during cirrhosis.

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Introduction

Cirrhosis of the liver is associated with extra-hepatic complications such as portal hypertension, hyperdynamic circulation, cardiomyopathy as well as renal dysfunction (1-5). The pathogenesis of these complications has been the focus of active research during last two decades. It is well known that nitric oxide (NO) is mechanistically linked to the pathophysiology of these complications (2,6). Many investigators have shown that impaired NO synthesis in hepatic sinusoids leads to increased portal pressure in patients as well as experimental models of cirrhosis (7-12). Paradoxically, systemic cardiovascular complications of cirrhosis (e.g. hyperdynamic

circulation and cardiomyopathy) are related to increased production of NO in the systemic circulation (13,14). Thus, previous studies have shown that NO synthase inhibitors can improve hyperdynamic circulation as well as cardiomyopathy in rats with cirrhosis but they may exacerbate hepatic resistance in experimental cirrhosis (15-17). This paradoxical effect of NO makes it a difficult therapeutic target in the management of cirrhosis.

It is currently known that NO is the gaseous byproduct of L-arginine conversion into L- citrulline which is catalyzed by NO synthase (NOS) in many cells and tissues. NOS exist in three isoforms: neuronal (nNOS), inducible (iNOS) and endothelial constitutive (eNOS). A huge number of experimental studies have

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demonstrated enhanced production of NO, through the activation of both endothelial and inducible isoforms of NOS in cirrhosis (16). Recent studies have shown that NO production can be affected by non-enzymatic NO production called entero-salivary circulation that sequentially relies on excretion of nitrate into saliva and reduction of salivary nitrate into nitrite by anaerobic buccal bacteria (18). The acidic gastric fluid of stomach reduces nitrite (swallowed with saliva) to NO under the acidic environment (18-20). Since gastric intraluminal NO is primarily absorbed into portal circulation and subsequently enters the liver sinusoids, it could be a target to enhance intra-hepatic NO levels to compensate for NO deficiency in hepatic sinusoids. Previous studies have shown that plasma, exhaled air and ascitic fluid were collected from patients with liver cirrhosis caused by different etiologies have demonstrated elevated levels of either NO or NO metabolites (21-23). Since there is no convincing data on salivary concentration of nitrite/nitrate and salivary pH in patients or experimental models of cirrhosis, this study is therefore aimed to investigate salivary levels of NO metabolites as well as pH and electrolyte composition in an experimental model of biliary cirrhosis.

Materials and Methods

Animals

The study was performed on sixteen 12 week-old male Wistar-Albino rats, with an initial weight at 250-280g. Animals were kept under constant temperature and humidity in a 12 hours controlled dark/light cycle and were allowed free access to standard rat chow and water. All animal procedures were in accordance with "Guide for the Care and Use of Laboratory Animals" (NIH US publication No 85-23, revised 1985) recommendations and the local ethics committee of Tehran University of Medical Sciences. There were two groups: Cirrhosis and Sham operated rats. Six to eight rats were used in each experimental group. For induction of cirrhosis, male rats underwent bile duct ligation (BDL) as described before (24). In brief, after induction of general anesthesia with intraperitoneal injection of ketamine (50 mg/Kg) and xylazine (5 mg/Kg), animals underwent laparotomy in which the bile duct was ligated by placing two double knots proximally and distally. The part of the bile duct between the 2 double knots was then excised. Sham-operated animals were used as a control.

Saliva collection

Twenty-eight days after the operation, animals were

anesthetized with pentobarbital (50 mg/Kg, i.p. injection). Cannulating the trachea was performed by a polyethylene tube to locate them on an inclined board and prevent apnea caused by pentobarbital. Submandibular ducts were cannulated with polyethylene microtube (PE10), as described by Yoshida *et al.*, (26,27). Pilocarpine (8 mg/Kg) was injected to peritonea as secretagogue. After 30 min, saliva was collected. Before freezing it at -80°C, pH was measured by a digital pH meter (Orion-520A, Thermo Electron Corporation, USA). Submandibular glands were then removed and snap frozen in liquid nitrogen for extraction of RNA. In a separate group of BDL and sham animals, the salivary glands were fixed in buffered formaldehyde solution (10%) for histological assessment. After cardiac puncture, sera were obtained from collected blood by centrifugation at 3000 x g for 10 min (4°C). Spleen and liver were also removed and weighed. The liver samples were obtained for histological assessment and confirmation of biliary cirrhosis.

Biochemical measurements

Total protein content of saliva samples were determined by Bradford method. Na⁺, K⁺, Mg⁺⁺, Ca⁺⁺ and phosphate levels in saliva and serum were assessed using an autoanalyzer system (Dr. Shariati Hospital Central Laboratory, TUMS). Determination of both serum and salivary amylase activity performed using a colorimetric method (Pars Azmoon enzyme assay kit). Concentration of salivary and serum nitrite (NO₂-) and nitrate (NO₃-) levels were determined using Griess method.

mRNA extraction and reverse transcription polymerase chain reaction (RT-PCR) procedure

Expression of eNOS, nNOS, iNOS, were assessed using real time RT-PCR in frozen submandibular glands taken from BDL or sham operated rats. Total RNA was extracted from tissue homogenate using RNase Fibrous Tissue mini kit (QIAGEN, Germany) according to the manufacturer's protocol. Further elimination of genomic DNA was performed by DNase (QIAGEN) during RNA extraction. For RT-PCR experiments, RNA was converted to cDNA using reverse transcriptase and oligo(dT)₁₆ primer according to the supplier's instructions (Aryatous biotech co., Iran). PCR reactions comprised of 2 µl of cDNA template, 10 pmol each of forward and reverse oligonucleotide primers, 10 µl of optimized PCR mastermix (with sybr green, Ampliqon, Denmark) in a total reaction volume of 20 µl. After initial 15 min incubation at 94 °C, PCRs were performed

using a 1 min annealing step, followed by a 1 min elongation step at 72 °C and a 1 min denaturation step at 94 °C. Forty PCR cycles were performed for amplification of above mentioned products. The level of transcriptional difference between cirrhotic rats and control group was calculated relative to the level of 18S ribosomal RNA expression. Oligonucleotide primers used for PCR amplification of rat nNOS, iNOS, eNOS and 18S ribosomal RNA (as housekeeping gene) were as follows:

1. Rat nNOS, Forward: 5'- TGG CAA CAG CGA CAA TTT GA, Reverse: 5'- CAC CCG AAG ACC AGA ACC AT.

2. Rat iNOS, Forward: 5'- ACC CAA GGT CTA CGT TCA AGA CA, Reverse: 5'- CAC ATC CCG AGC CAT GC.

3. Rat eNOS, Forward: 5'- CCG GCG CTA CGA AGA ATG, Reverse: 5'- CAG TGC CAC GGA TGG AAA TT.

4. Rat 18S ribosomal RNA, Forward: 5'- ATC ACC TTT CGA TGG TAG TCG, Reverse: 5'- TCC TTG ATG TGG TAG CC.

Histological studies

Liver and submandibular tissues from control and cirrhotic rats were prepared for light microscopy evaluation. Paraffin embedded blocks were prepared and sectioned in 5 µm thickness; the sections were stained with Hematoxylin and Eosin. Trichrome staining was also used for liver samples.

Statistical analysis

Results are expressed as mean ± SEM. Multiple statistical comparisons were made using Student's unpaired *t*-tests. Differences between non-normally distributed variables were examined by non-parametric Mann-Whitney U test. *P*-values less than 0.05 were considered statistically significant. GraphPad Prism software was used to perform statistical analysis.

Results

Four weeks after bile duct ligation animals revealed manifestations of biliary cirrhosis such as jaundice, dark urine, splenomegaly as well as raise in plasma alkaline phosphatase (928.8 ± 103.6 in BDLs vs 398.8 ± 35.99 in sham-operated rats, $P < 0.05$). Trichrome staining of liver tissue in BDL rats revealed the fibrous expansion of portal areas with a marked bridging configuration to marked fibrosis with vaguely nodular configuration. Bile duct proliferation and mild spotty necrosis of hepatocytes without confluent necrosis were also identified in BDL rats.

The saliva was collected from BDL and sham-operated control rats and the level of salivary pH, electrolytes, total protein and amylase activity are shown in Table 1. There was no significant difference in salivary Na⁺, Mg⁺⁺, phosphate, total protein concentrations or salivary amylase activity between control (sham) groups and cirrhotic (BDL) animals. Salivary pH was however significantly lower in BDL rats in comparison with sham-operated animals ($P < 0.05$). K⁺ and Ca⁺⁺ levels also showed a significant reduction following induction of cirrhosis (Table 1).

Table 1. Salivary pH, electrolytes, total protein concentration and amylase activity in bile duct ligated (BDL), and sham-operated (SHAM) control rats. Values are the values Mean ± SEM, n=6-8 in all experiments. * $P < 0.05$ in comparison with SHAM group.

	SHAM	BDL
Salivary pH	8.81 ± 0.01	8.62 ± 0.11*
Na ⁺ (mg/mL)	46.93 ± 4.17	51.29 ± 10.60
K ⁺ (mg/mL)	32.69 ± 1.03	25.50 ± 1.74 *
Mg ⁺⁺ (mg/dL)	2.00 ± 0.12	1.83 ± 0.21
Ca ⁺⁺ (mg/dL)	1.41 ± 0.08	0.99 ± 0.08 *
Phosphate (mg/dL)	1.37 ± 0.20	1.13 ± 0.16
Total protein (mg/mL)	4.23 ± 1.00	4.19 ± 0.65
Amylase activity (IU)	14517 ± 13111	10445 ± 8820

Figure 1A shows salivary nitrite and nitrate levels in control and cirrhotic rats. We observed a statistically significant increase in salivary levels of nitrite as well as nitrate in BDL rats ($P < 0.05$ for nitrite and $P < 0.01$ for

nitrate levels). This increase in salivary nitrite and nitrate level in BDL rats was also observed in plasma samples as shown in Figure 1B. We used real time RT-PCR to estimate eNOS, nNOS and iNOS mRNA levels

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in the submandibular gland and the results are shown in Figure 2. The relative transcriptional level of eNOS showed a significant reduction in submandibular glands of cirrhotic rats ($P=0.05$). While nNOS mRNA did not show a statistically significant difference between control and cirrhotic animals (Figure 2B). We observed the very little amount of iNOS expression in the submandibular gland of sham-operated and BDL rats which did not show a significant difference between groups (Figure 2C).

There was no significant difference in the relative weight of submandibular gland (normalized with total rat body weight) between cirrhotic and control group (0.0008292 ± 0.00004044 vs. 0.0008160 ± 0.00002245). The microscopic study showed mixed normal acinar cells (mucous and serous cells) and no vacuolization in submandibular glands taken from BDL rats. Ductal structures were also normal, and connective tissue showed normal appearance with no edema, atrophy, leukocytic infiltration or fibrotic changes (Figure 3).

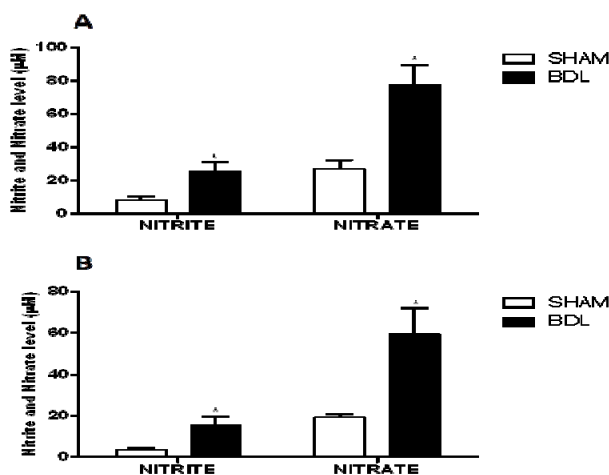


Figure 1. Salivary (A) and plasma (B) nitrite and nitrate level (μM) in bile duct ligated (BDL), and sham-operated (SHAM) rats.

* $P < 0.05$, ** $P < 0.01$

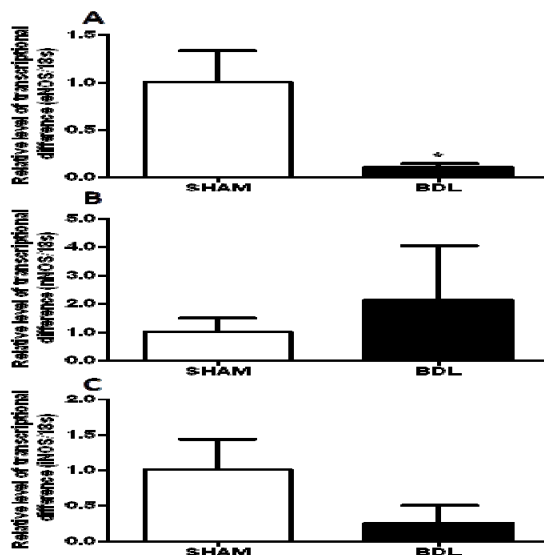


Figure 2. The effect of bile duct ligation on the expression of eNOS (A), nNOS (B) and iNOS (C) in rat submandibular gland. Real time RT-PCR was used to assess the relative level of transcriptional difference between control and cirrhotic rats. 18S ribosomal RNA was used as a housekeeping gene. Data are expressed as mean \pm SEM. * $P=0.05$ in comparison with controls.

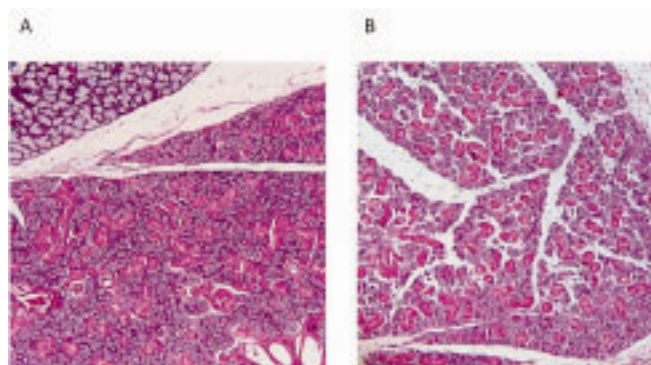


Figure 3. Histological assessment of submandibular gland from sham operated (A) and bile duct ligated (B) rats using H&E staining (x 100)

Discussion

Most of previous reports on composition of saliva in patients with cirrhosis are focused on finding markers for early diagnosis of primary biliary cirrhosis and so far there is a little knowledge about entero-salivary circulation of NO metabolites in patients or experimental cirrhosis. There is evidence to show that salivary excretion of nitrite and nitrate is important in the non-enzymatic production of NO in the gut (20). Since, NO plays an important role in pathogenesis of portal hypertension in cirrhosis, we carried out a study to measure salivary levels of nitrate and nitrite as well as pH and electrolyte composition in an experimental model of biliary cirrhosis.

Many studies have shown the elevation of plasma nitrite and nitrate levels in cirrhosis (3). Current results corroborated with these findings and additionally, we assessed salivary nitrate and nitrite levels in rats with cirrhosis which showed elevated salivary excretion of nitrate and nitrite in BDL rats. Hence, it has been shown that salivary nitrate/nitrite levels are increased in cirrhotic rats while there was no elevation in the mRNA expression of nNOS, eNOS, and iNOS in submandibular glands of cirrhotic groups. This indicates that an increased salivary level of nitrite/nitrate is less likely to be linked to increased enzymatic production of NO in the salivary epithelium. NO is synthesized in salivary epithelium mainly by an eNOS-dependent mechanism, and eNOS expression was significantly decreased in submandibular tissue obtained from cirrhotic rats. Although we did not measure eNOS protein level or eNOS enzymatic activity in a submandibular gland in cirrhotic animals, reduced mRNA expression of eNOS in cirrhosis is against the exaggerated enzymatic production of NO in the salivary epithelium. Thus, increased salivary nitrate/nitrite does not necessarily

reflect enhanced production of endogenous NO in the submandibular gland. In contrast, the entero-salivary cycle of nitrate/nitrite may play a role in this phenomenon. In other words, nitrate/nitrite can be transported from the blood stream by submandibular glands and excreted into saliva, and this mechanism may have been exaggerated during cirrhosis.

Regardless of the reason that leads to elevation of NO metabolites in the saliva, this alteration can potentially exaggerate entero-salivary circulation of nitrate/nitrite. NO which is produced from swallowed salivary nitrite in the stomach could enter to the portal vein and subsequently cycles through liver sinusoids. Since intra-hepatic NO production is impaired in cirrhosis, this intrahepatic circulation of NO might play a beneficial role in cirrhotic liver and may affect intrahepatic resistance as well as portal pressure. We did not measure the portal pressure after oral ingestion of dietary nitrate in cirrhotic rats, but it can be investigated in future experiments. This is potentially important since NO has a paradoxical effect on intrahepatic versus systemic circulation (13,14). Although intra-hepatic production of NO is decreased in cirrhosis, pharmacological NO donors show the adverse effect in these patient populations due to decreasing systemic vascular resistance. Enhanced entero-salivary circulation of nitrate/nitrite could be associated with the more specific delivery of NO to hepatic tissue and could be the target to manage the double-edged sword effects of NO in the pathogenesis of cirrhotic cardiovascular phenomena.

In addition to salivary nitrate/nitrite, we assessed salivary pH as well as some electrolytes. As shown in Table 1, measurement of salivary pH in cirrhotic rats showed lower pH in comparison to control group. Besides, K⁺ level decreased in cirrhotic rats while, no changes was observed in salivary Na⁺. Based on these

data, it is likely that salivary ion transport/exchange is impaired in cirrhotic rats. Thus, these alterations could be attributed to changes in either expression or activity of some ion carriers which involve in the regulation of cell pH, cell volume, tonicity, and intracellular Cl⁻ and H⁺ homeostasis. Cl⁻/HCO₃⁻ exchanger (SLC4), Na⁺/H⁺ exchanger (NHE) are in cooperation with each other to regulate the cell content of bicarbonate, chloride and hydrogen to maintain the cell homeostasis of these ions and salivary compound. NHE extrudes hydrogen ions, thereby generating intracellular bicarbonate in cooperation with carbonic anhydrase. SCL4 extrudes bicarbonate into the saliva according to the intracellular pH, the intracellular concentration of HCO₃⁻, and the Cl⁻ gradient (27-31). In a preliminary study, we investigated the expression of these exchangers in submandibular glands by real time PCR. There were no statistically significant changes in mRNA expression of SLC4 and NHE in the submandibular gland of cirrhotic rats (data not shown). Future studies can investigate other possibilities which lead to disturbing the salivary compound.

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