Effect of L-NAME on nitric oxide and gastrointestinal motility alterations in cirrhotic rats

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Abstract

AIM: To investigate the effect of L-NAME on nitric oxide and gastrointestinal motility alterations in cirrhotic rats.

METHODS: Rats with cirrhosis induced by carbon tetrachloride were randomly divided into two groups, one (n=13) receiving 0.5 mg·kg⁻¹·day⁻¹ of N·G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, for 10 days, whereas the other group (n=13) and control (n=10) rats were administrated the same volume of 9g·L⁻¹ saline. Half gastric emptying time and 2h residual rate were measured by SPECT, using 99mTc-DTPA-labeled barium sulfate as test meal. Gastrointestinal transition time was recorded simultaneously. Serum concentration of nitric oxide (NO) was determined by the kinetic cadmium reduction and colorimetric methods. Immunohistochemical SABC method was used to observe the expression and distribution of three types of nitric oxide synthase (NOS) isoforms in the rat gastrointestinal tract. Western blot was used to detect expression of gastrointestinal NOS isoforms.

RESULTS: Half gastric emptying time and trans-gastrointestinal time were significantly prolonged (124.0±26.4min; 33.7±8.9min; P<0.01), (12.4±0.5; 9.5±0.3h; P<0.01), 2h residual rate was raised in cirrhotic rats than in controls and cirrhotic rats treated with L-NAME (54.9±7.6%, 13.7±3.2%; 34.9±10.3%, P<0.01). Serum concentration of NO was significantly increased in cirrhotic rats than in the other groups (8.20±2.48μmol·L⁻¹, 5.94±1.07μmol·L⁻¹, and control (5.66±1.60)μmol·L⁻¹, P<0.01. NOS staining intensities which were mainly located in the gastrointestinal tissues were markedly lowered in cirrhotic rats than in the controls and cirrhotic rats after treated with L-NAME.

CONCLUSION: Gastrointestinal motility was remarkably inhibited in cirrhotic rats, which could be alleviated by L-NAME. Nitric oxide may play an important role in the inhibition of gastrointestinal motility in cirrhotic rats.
Total gastrointestinal transition time (TGIT) After isotopic scanning, the time of BaSO₄ excretion through anus was recorded.

Alterations of NO and NOS

Assay of serum NO₂⁻/NO₃⁻-content Rats were decapitated and the blood was collected and quietly placed for 60 min. After 4000r·min⁻¹ centrifugation for 15 min, the supernatant was used for NO₂⁻/NO₃⁻-measurement. Operation was performed following the instructions of the reagent kit (purchased from Institute of Nuclear Medicine, General Hospital of Chinese PLA).

Expression of NOS in gastrointestinal tract Immunohistochemical method was used with immunohistochemical reagents from Wuhan Boshide Biotech Co Lt. Five rats were randomly selected from each of the three groups. Each rat was anesthetized with 10g·kg⁻¹ sodium pentobarbital (50mL·kg⁻¹) through abdominal cavity injection and perfused with 40g·L⁻¹ polyformaldehyde for 1.5h. Gastric, small intestinal and colonic tissues were immediately taken out and immersed in 200g·L⁻¹ sucrose solution at 4°C for 24 h until the tissues sank to the bottom. Then the tissues were cryomolded at -20°C into slices 14-16µm thick. The tissues were rinsed three times with 0.01mmol·L⁻¹ PBS for 5 min; treated with peroxide and methanol for 15 min; vibrated and washed with PBS for 5 min three times; and blocked with normal bovine serum for 30 min. Rabbit anti-NOS-1 (1:100 rabbit polyclonal antibody), anti-NOS-2 (1:50 rabbit polyclonal antibody), anti-NOS-3 (100 rabbit polyclonal antibody) were added and the solution was incubated at 4°C overnight. Then it was vibrated and washed with PBS three times for 5 min. Biotinized sheep-anti-rabbit IgG was added and the solution was let react for 2 h at RT. The sample was then rinsed in TBS+1g·L⁻¹ NP-40 10min×5 and developed with DAB.

Statistical analysis

Analysis of variance was conducted using NOSA statistics program (Fourth Military Medical University), and the results were presented in form of ±x.
Alterations of NO and NOS

Serum concentration of NO The serum NO\textsubscript{2}-/NO\textsubscript{3}- concentrations were (8.20±2.48) µmol·L\textsuperscript{-1}, (5.94±1.07) µmol·L\textsuperscript{-1}, and (5.66±1.60) µmol·L\textsuperscript{-1} in the rats of untreated group, normal control group, and treated group, respectively. It was apparent that NO concentration in the untreated group was significantly higher than in other groups (P<0.01).

Expression of NOS in rat gastrointestinal tract NOS immunohistochemical staining showed that NOS\textsubscript{1}, NOS\textsubscript{2}, and NOS\textsubscript{3} had similar distribution in gastrointestinal mucosal lamina propria layer, principally in neutrophiles, monocytes, macrophages and some lymphocytes of gastrointestinal mucosal lamina propria layer.
interstitial. NOS existed mainly in intermuscular nerve bundles in the gastrointestinal wall, endocrine cells in the mucosal layer, macrophages in gastrointestinal mucosal lamina propria layer interstitial, and some lymphocytes. In normal rats, NOS positive cells were mainly located in the lower third part of the gastric mucosal layer, in the intermuscular nerve bundles and villus interstitial of small intestine, and pervasively in colonic mucosal villus interstitial (Figure 4). In cirrhotic rats, NOS positive cells decreased significantly in the whole gastrointestinal tract and intermuscular nerve bundle. These two indexes in cirrhotic rats treated with L-NAME were significantly higher than those in untreated rats.

**Western blot analysis**  Protein electrophoresis showed that the sampling amounts of gastric, small intestinal and colonic proteins of rats in the three groups were the same, and that the protein composition in the small intestine was quite different from that in the stomach and the colon. Western blot showed that NOS expression decreased significantly in the gastric and colonic tissues of cirrhotic rats, and it returned to normal after treated with L-NAME. NOS was not detected in the small intestine in either groups.

![Western blot analysis](image)

**Figure 5** Expression of NOS in stomach(1,2,3), intestine(4,5,6) and colon(7,8,9) of rats 1,4,7: Control; 2,5,8: Cirrhotic; 3,7,9: Treated

**DISCUSSION**  NO plays an important role in gastrointestinal physiological activities as well as in the pathogenesis and progress of many severe diseases[34-40]. It is involved in the regulation of gastrointestinal smooth muscle contraction and secretion of water and salt of intestinal epithelial cells [41-45]. It mediates endotoxin-induced inhibition of gastric acid secretion, protects gastrointestinal mucosa, sustains mucosal blood flow, inhibits neutrophile adhesion to vascular endothelium and blocks platelet adhesion; prevents macrophage activation. NOS is the rate-limiting enzyme of NO synthesis, which exists pervasively in gastrointestinal tissues, including epithelia, fibroblasts, macrophages, inherent and infiltrating lymphocytes, neutrophilies, monocytes, smooth muscle cells, endocrine cells, and intramuscular ganglia. The kinds and densities of NOS positive cells diverse at different regions [34-40]. NOS can be classified into 3 types according to biological characters and encoding genes: neuronal type nNOS (NOS1), endothelial type eNOS (NOS3) and induced type iNOS (NOS2). There are 50% homology between them. NOS1 primarily exists in neural and epithelial cells. NOS2 was firstly separated from macrophages and later discovered to exist in other kinds of cells such as vascular smooth muscle cells. NOS3 mainly exists in vascular endothelial cells. According to the activity dependence on Ca\(^{2+}/CaM\), NOS has two subtypes: constitutive NOS (cNOS), including NOS1, and NOS3, whose activity is regulated by Ca\(^{2+}/CaM\), and induced NOS, including NOS2 whose enzymatic activity is not dependent on Ca\(^{2+}/CaM\) but needs inducing factors. The cNOS primarily exists in normal vascular endothelial cells, and is also found in adrenal gland cells, platelets, fibroblasts, PMNs, brain cells and certain non-cholinergergic, non-adrenalergenic synapses [41-43].

NOS expression in gastrointestinal tissues differs in certain pathological situations. In abdominal inflammation, positive cells on the small intestinal wall mainly exist in the mucosal lamina propria layer, over 80% of the positive cells are CD45 positive inflammatory cells, about 15% are CD3 positive T lymphocytes, and epithelial cells are all negative. In ulcerative colitis, iNOS positive cells are mainly intestinal epithelial cells, while mucosal inherent cells are all negative. The status in cirrhosis is not known yet [46-48]. NO is the major inhibitory neurotransmitter released by non-adrenaline, non-cholinergergic neurons, which is closely related to gastrointestinal motility and pathology. Gastric physiological expansion and intestinal peristalsis are regulated by NO, which can directly inhibit gastrointestinal smooth muscle contraction and retard gastrointestinal motility. NOS inhibitor can promote ascites re-absorption and urinary sodium excretion of cirrhotic rats, and the rats’ colonic motility recovery after abdominal operations. We prepared a toxic cirrhotic rat model, used radioactive isotopic method to determine gastric emptying functions of the rats, and recorded the total gastrointestinal transitional time (TGIT). The results showed that TGIT of the rats in untreated group was significantly longer than that of the rats in the control group and the treated group, while gastric emptying was significantly slower in the former. It suggested the dysfunction of gastrointestinal motility in cirrhosis.

Cirrhotic patients are prone to develop endotoxemia due to flora translocation, enhanced absorption of endotoxins and reduced hepatic detoxification. Endotoxins stimulate vascular endothelial cells, activate NOS, and consequently increasing NO synthesis. The serum NO concentration in cirrhotic patients rose significantly [46-48], and the same findings were observed in cirrhotic rat’s model in our experiment. However, immunohistochemical staining revealed the different distribution of NOS1, NOS2 and NOS3 as described above. The quantity of NOS positive cells in cirrhotic rat gastrointestinal tissues was significantly lower than that in rats treated with L-NAME and normal control, so was NOS staining intensity in nerve bundles. Western blot was used to examine the expression of the three types of NOS in gastric, small intestinal and colonic tissues of rats and the same results were obtained as we did through NOS immunohistochemistry. These results indicate that local synthesis of NO is regulated by many factors in cirrhotic rat gastrointestinal tissues [49].

NOS-specific competitive inhibitor L-NAME was used to treat cirrhotic rats and the results were as follows: TGIT of untreated cirrhotic rats was significantly longer than that of normal or treated cirrhotic rats, and gastric emptying in the former group was significantly slower. L-NAME treatment significantly accelerated gastric emptying and reduced TGIT. The serum NO concentration in cirrhotic rats was elevated, and L-NAME treatment reduced the serum NO concentration and gastrointestinal NO synthesis as well. These results indicate that NO contributes greatly to cirrhotic gastrointestinal motility dysfunction. NOS inhibitor L-NMMA can reduce the duration of small intestinal digestive interval MMC I phase as well as the total duration of MMC, whereas the occurrence frequency of MMC was raised and small intestinal motility was enhanced. This might be one of the mechanisms of L-NAME enhancing cirrhotic rat gastrointestinal motility [49].

Disorder of cirrhotic gastrointestinal motility is a multi-factor disease. Our research showed that the gastrointestinal motility of cirrhotic rats was significantly inhibited, which was demonstrated by slowed gastric emptying and prolonged gastrointestinal transition time. As NO activity in the serum and tissues of cirrhotic rats was comparatively high, we used NOS-specific inhibitor to treat the rats and removed such inhibition, and found that NO played an important role in cirrhotic gastrointestinal motility dysfunction. Thus, we conclude that drugs inhibiting NO synthesis would be clinically conducive to alleviate the gastrointestinal motility dysfunction of cirrhotic patients and could consequently reduce the occurrence of cirrhosis-related complications.

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