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World J Gastroenterol 2002;8(2):328-332
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

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

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Received 2001-08-23 **Accepted** 2001-09-05

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 0.5mg·kg^{-1} per day 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 ^{99m}Tc-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;72.1±15.3min; *P*<0.01), (12.4±0.5h; 9.5±0.3h; 8.2±0.8h; *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±2.48)μmol·L^{-1}, (5.94±1.07) μmol·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 lower 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.**

Wang X, Zhong YX, Zhang ZY, Lu J, Lan M, Miao JY, Guo XG, Shi YQ, Zhao YQ, Ding J, Wu KC, Pan BR, Fan DM. Effect of L-NAME on nitric oxide and gastrointestinal motility alterations in cirrhotic rats. *World J Gastroenterol* 2002;8(2):328-332

INTRODUCTION

Investigation in gastrointestinal motility has promoted the interest of researchers in the functional changes in gastrointestinal motility of cirrhotic patients[1-8]. It is validated that nitric oxide (NO) plays a pivotal role in neural transduction and gastrointestinal motility regulation as a neurotransmitter and messenger molecule with various physiological functions[9-19]. However, the relationship between NO and functional changes in cirrhotic gastrointestinal motility is not yet clear[20-27]. We used nitric oxide synthase (NOS) -specific inhibitor to treat cirrhosis model rats and observe changes in their gastrointestinal motility so as to disclose the role of NO in such changes and to provide experimental basis for diagnosis of cirrhotic gastrointestinal motility abnormalities.

MATERIALS AND METHODS

Preparation of animal model

Male SD rats were provided by the Experimental Animal Center, the Fourth Military Medical University, weighting (250±50)g, fed with standard granule food, and randomly divided into model group(26 rats) and normal control group (10 rats). CCl_4 toxic cirrhosis model preparation: rats in the model group received subcutaneous injection of CCl_4 (3mL·kg^{-1}) twice a week for 12 weeks; and rats in the control group received olive oil (3mL·kg^{-1}) twice a week for 12 weeks. At the end of 12 weeks, cirrhosis model group was randomly subdivided into treated group and untreated group. Normal SD rats were served as controls, each group having 10 rats. Rats in the treated group were given 0.5mg·kg^{-1}d^{-1} N_G-nitro-L-arginine methyl ester (L-NAME, Sigma product, USA), a NOS inhibitor, through intragastric administration for 10 days, and the untreated group and control group received gastric delivery of 9g·L^{-1} NaCl once a day for 10 days. Hepatic tissue samples were normally fixed with 40g·L^{-1} polyformaldehyde, prepared into paraffin wrapped sections, stained with HE and observed under light microscope.

Analysis of gstrointestinal motility

Examination of gastric emptying Experimental animals were fast for over 12h without receiving drugs or food that might influence gastric motility during the previous 2 weeks.!Each rat received 2mL $BaSO_4$ mixed with 3.7GBq·L^{-199m}Tc-DTPA through the esophagus into the stomach in 2min, and was placed lying on the back. SPECT (Type CT, Sophycamera Co, France) detector was focused on the abdomen of the animals with the whole stomach as the Region of Interest (ROI); radioactivity was recorded and images were displayed for 60s as the total radioactivity. Gastric images were then taken at 15, 30, 45, 60, 90 and 120min, delineating the ROI; the curve of time-half gastric emptying was drawn and gastric residual radioactivity rates at different time points were calculated by computer.

Total gastrointestinal transition time (TGIT) After isotopic scanning, the time of $BaSO_4$ excretion through anus was recorded.

Alterations of NO and NOS

Assay of serum NO_2-/NO_3-content Rats were decapitated and the blood was collected and quietly placed for 60min. After 4000r·min^{-1} centrifugation for 15min, the supernatant was used for NO_2-/NO_3-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 Ltd. Five rats were randomly selected from each of the three groups. Each rat was anesthetized with 10g·L^{-1} sodium pentabarbitol (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℃ for 24h until the tissues sank to the bottom. Then the tissues were cryotomized at -20℃ into slices 14-16μm thick. The tissues were rinsed three times with 0.01mmol·L^{-1} PBS for 5min; treated with peroxide and methanol for 15min; vibrated and washed with PBS for 5min three times; and blocked with normal bovine serum for 30min. 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℃ overnight. Then it was vibrated and washed with PBS three times for 5min. Biotinized sheep-anti-rabbit lgG was added and the solution was let react at 37℃ for 30min. The system was washed again with 0.01mmol·L^{-1}PBS for 5min×3; SABC was added and let react at 37℃ for 30min; and washed with 0.01mmol·L^{-1}PBS for 5min×4. The stain was developed with DAB and stained with lignin. The sample was normally dehydratized till transparent and sealed with neutral resin. Microscopic observation was done and photo taken under microscope.

Determination of NOS in gastrointestinal tract with Western blot Animals fasted 12h before experiment without water deprivation. Three rats each from treated group, untreated group and control group were randomly chosen, decapitated, and eviscerated immediately to obtain the stomach, small intestine and colon, which were rinsed in ice-water containing 0.1mmol·L^{-1}PMSF, frozen in liquid nitrogen and moved into -70℃ refrigerator for preservation. The whole process should be finished within 5min. Tissue lysis liquid was prepared with ion-free water containing 0.1mmol·L^{-1}PMSF. The gastrointestinal tissues were weighed and homogenized (3500r·min^{-1}, 5s×5) in ice-bathing, the mass/volume ratio of tissue to tissue lysis liquid being 1∶5. Centrifugation was then performed at 12500r·min^{-1} for 10min; the supernatant was separated and preserved at -70℃; the protein concentration in the extract was measured by the Bradford method.

Eighty g·L^{-1} PAGE gel was prepared and 2×SDS loading buffer was added to the protein samples, followed by heating at 100℃ for 3min. Centrifugation was performed again and protein samples of the same amount were added. Electrophoresis was done with 20mA current; and the gel was stained with Coomassie brilliant blue. Separation of protein extracts of the stomach, small intestines and colons of the rats was accomplished with 80g·L^{-1} SDS-PAGE. After electrophoresis, electric transfer of the proteins onto NC membrane was done with a constant current of 0.8mA·cm^{-2} for 1h using semi-dry electric transfer device (Beijing 61 Factory). Transfer buffer ingredients (25mmol·L^{-1}Tris-Hcl, 192mmol·L^{-1} glycine, 10g·L^{-1}SDS, 200g·L^{-1} methanol, pH8.3. TBS pH7.5+50g·L^{-1} non-fat milk+0.5g·L^{-1} NP-40) were used for blocking the sample for 2h at RT. Primary antibody NOS_1, NOS_2 and NOS^a-3 (1∶100 rabbit multiclonal antibody) was diluted with TBS buffer containing 1g·L^{-1}BSA and added. The sample was incubated at 4℃ for 16-18h and rinsed in TBS three times for 10min. HRP linked sheep-anti-rabbit secondary antibody (1∶400, Boshide Co.) was diluted with TBS, added to the sample and let react for 2h at RT. The sample was then rinsed in TBS+1 g·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 $\bar{x}\pm s$.

RESULTS

Establishment of rat model with cirrohosis

Model rats had hepatomegaly and splenomegaly, the liver became hard, the edge turned blunt, and the surface was not smooth, with nodules of varied sizes. Light microscopy showed hepatocyte regeneration, fat degeneration, proliferation of collagen fibers, and pseudo-lobulation.

Gastrointestinal motility

Gastrointestinal motility for barium Gastric semi-solid emptying: after intragastric administration of $BaSO_4$ containing ^{99m}Tc-DTPA, abdominal radioactivity images were taken in 8 rats in normal group (Figure 1A), untreated group (Figure 1B) and treated group (Figure 1C) each at 5, 30, 90 and 120min. The images showed that gastric emptying of cirrhotic rats was slowed down, and after treatment it was accelerated. The residual rate of gastric semi-solid substance of rats was observed dynamically.

Gastric emptying of cirrhotic rats was slowed down, the 2h residual rate being increased significantly. After L-NAME treatment, the gastric emptying was accelerated, the 2h residual rate decreased significantly (Figure 2 and Table 1).

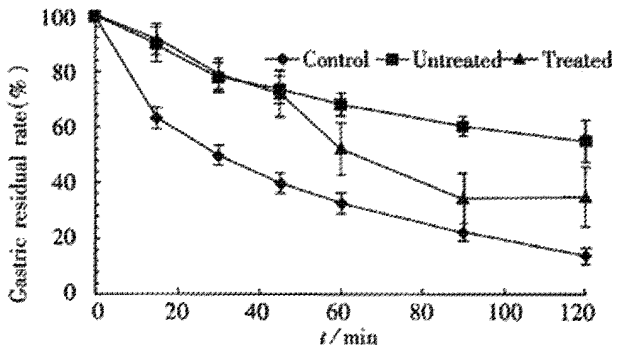

Figure 2 Residual rate curves of gastric semi-solid substance of rats

Table 1 Half-emptying time and 2h residual rate (n=8)

Groups	$GET_{1/2}$(min)	RR2h(%)
Control	33.7	13.7
Untreated	124.0	54.9[b]
Treated	72.1	34.9

[b]P<0.01, *vs* control and treated. $GET_{1/2}$: half-emptying time; RR2h: 2h residual rate

Gastrointestinal transition time TGIT of rats in untreated group (12.4±0.5)h was significantly longer (P<0.01) than that in the control group (9.5±0.3)h, whereas TGIT of rats in treated group (8.2±0.8)h was significantly shorter (P<0.01). Figure 3 shows that gastrointestinal transition of barium sulfate apparently slowed down in cirrhotic rats, and was significantly accelerated after L-NAME treatment; and barium sulfate was excreted in 6h after delivery.

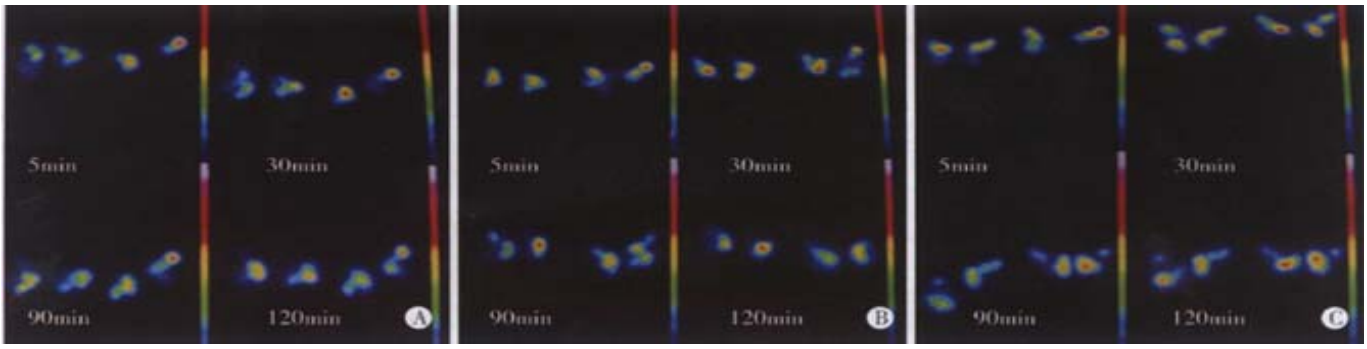

Figure 1 Gastric emptying for barium in rats. A: Normal; B: Cirrhotic; C: Cirrhotic treated with L-NAME

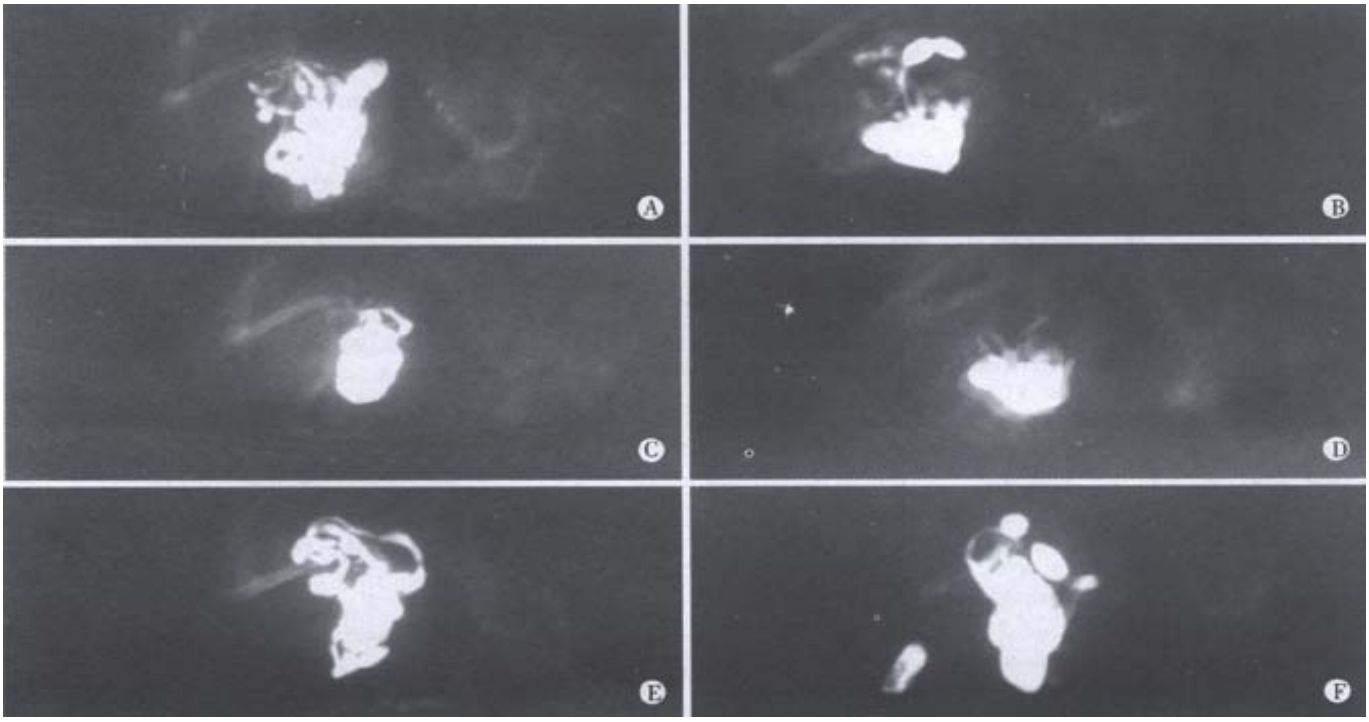

Figure 3 X-ray analysis of rat gastrointestinal motility.
A, B: Control rats at 5min and 6h; C, D: Untreated rats at 5min and 6h; E, F: Treated rats 5min and 6h

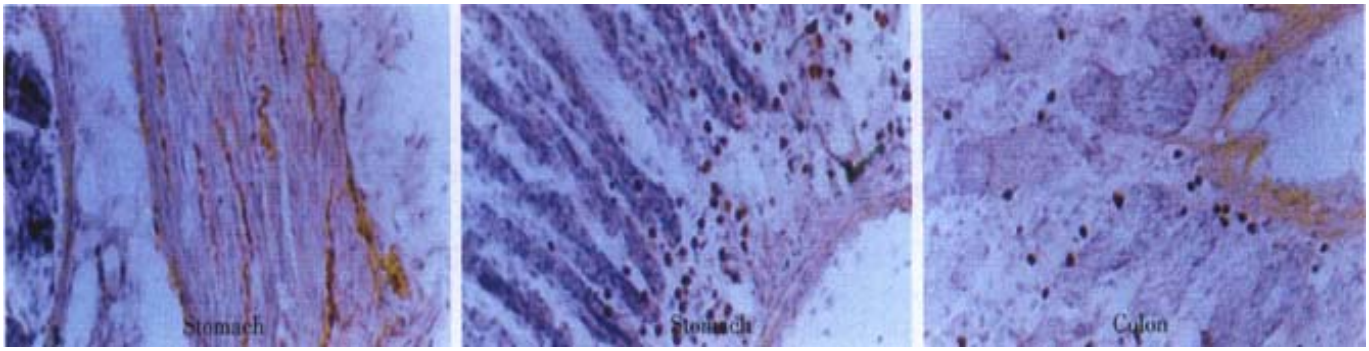

Figure 4 Expression and distribution of NOS in rat gastrointestinal tract with cirrhosis.

Alterations of NO and NOS

Serum concentration of NO The serum NO_2-/NO_3- concentrations were (8.20±2.48)μmol·L^{-1}, (5.94±1.07)μmol·L^{-1}, and (5.66±1.60)μmol·L^{-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_1, NOS_2 and NOS_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_1 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.

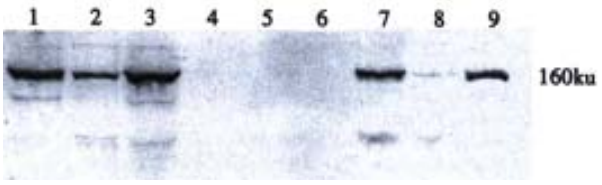

Figure 5 Expression of NOS_1 in stomach(1,2,3), intestine(4,5,6) and colon(7, 8,9) of rats 1,4,7: Control; 2,5,8: Cirrhotic; 3,7,9: Treated

DISCUSSION

NO plays an important role in gastrointestinal physiological activities as well as in the pathogenesis and progress of many severe diseases[27-31]. It is involved in the regulation of gastrointestinal smooth muscle contraction and secretion of water and salt of intestinal epithelial cells[32,33]. 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, neutrophiles, 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 (NOS_1), endothelial type eNOS (NOS_3) and induced type iNOS (NOS_2). There are 50% homology between them. NOS_1 primarily exists in neural and epithelial cells. NOS NOS_2 was firstly separated from macrophages and later discovered to exist in other kinds of cells such as vascular smooth muscle cells. NOS_3 mainly exists in vascular endothelial cells. According to the activity dependence on Ca^{2+}/CaM, NOS has two subtypes: constructive NOS (cNOS), including NOS_1 and NOS_3 whose activity is regulated by Ca^{2+}/CaM, and induced NOS, including NOS_2 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-cholinergenic, non-adrenalergenic synapses[41,42].

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 *coli*tis, iNOS positive cells are mainly intesitinal epithelial cells, while mucosal inherent cells are all negative. The status in cirrhosis is not known yet[43-45]. NO is the major inhibitory neurotransmitter released by non-adrenaline, non-cholergenic 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 transition 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 floratranslocation, 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 NOS_1, NOS_2 and NOS_3 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 sreum 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[50].

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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Edited by Ma JY