Original Article
A novel liver-targeted nitric oxide donor UDCA-Thr-NO protects against cirrhosis and portal hypertension

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Abstract: Portal hypertension (PHT) is a common liver disease that is closely related to cirrhosis and has a high morbidity and mortality. The present study aimed to probe the efficacy of a novel nitric oxide (NO)-releasing agent with NO linked to ursodeoxycholic acid (UDCA) through threonine (UDCA-Thr-NO) as a liver-targeted therapy for cirrhosis and PHT. After intraperitoneal treatment of dimethyl nitrosamine-induced cirrhotic rats for 3 or 4 weeks, UDCA-Thr-NO could prevent ascites formation and reduce portal pressure instead of carotid artery pressure, when compared with UDCA or compound embryonic bovine liver extract tablets. Biochemical analysis of the rat sera also revealed that UDCA-Thr-NO improved the levels of alanine aminotransferase and total bilirubin and reduced the level of hydroxyproline (P < 0.05). Colorimetric analysis of the liver tissue by staining hematoxylin-eosin (HE) and Sirius red (SR) showed that UDCA-Thr-NO could improve pathological changes and reduce liver collagen deposition and intrahepatic resistance without affecting systemic circulation. It was concluded that UDCA-Thr-NO had a protective effect on liver injury and could be utilized to improve cirrhosis and PHT.

Keywords: Cirrhosis, liver-targeted drug, nitric oxide, portal hypertension, UDCA, UDCA-Thr-NO

Introduction

Portal hypertension (PHT) is a major complication of late-stage liver diseases such as cirrhosis, which can lead to esophageal or gastric varices, rupture hemorrhage and other serious syndromes including gastrointestinal hemodynamics, ascites, and hepatopulmonary syndrome. Thereby, PHT is the main cause of death in cirrhotic patients [1, 2], whereas reducing the intrahepatic resistance is a principle method to treat PHT [3]. As reported, the reduced production of nitric oxide (NO) can contribute to high intrahepatic resistance in cirrhosis [4].

NO is a small, diffusible, and highly reactive molecule and the key substance mediating hepatic sinusoid resistance and liver microcirculation, and it can produce vasodilation and antihypertension effect. DeLeve et al showed a reduced level of NO in the hepatic sinusoids of cirrhotic patients, which led to the hepatic sinusoidal obstruction syndrome and higher intrahepatic pressure [5].

In the past few decades, many NO-donor drugs have been developed for PHT that showed some efficacy in animal and clinical trials, but the conventional NO donors still cannot meet the clinical demand due to their nonspecific *in vivo* distribution. This property of the conventional NO donors together with the paradoxical regulation of NO in PHT makes them to have not only unsatisfactory efficacy but also systemic adverse effects [6, 7]. Furthermore, NO is chemically reactive and has a wide range of biological activities but with an extremely short half-life *in vivo* (about 0.1-5 s) [8]. For these reasons, an ideal NO-donor drug or drug delivery system for treating PHT should directly deliver NO into the liver and act to decrease the intrahepatic resistance without affecting systemic vasodilatation [8, 9]. In addition, a quantitative NO release and the maintenance of an effective concentration of NO are also critical factors for NO-releasing drugs [10-12].

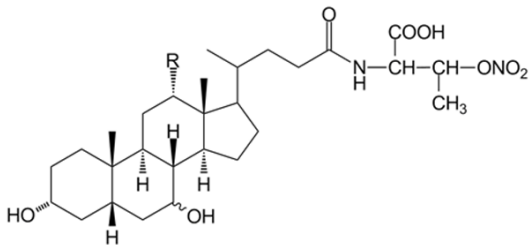Currently, many common hepatic-targeting carriers are available, including both macromolec-

Figure 1. The structure of UDCA-Thr-NO. A novel liver-targeted NO donor having NO conjugated with UDCA via a threonine spacer.

ular carriers, such as antibodies, asialoglyco-proteins and galactose albumin, and small molecules such as bile acids [13-15]. Among them, bile acids, which are almost exclusively synthesized in the liver, are the only molecules that have high oral availability and good biocompatibility. Therefore, using bile acids as the carrier to target liver has become an attractive direction for developing liver-targeting drugs [16-18].

NCX-1000 was the first liver-targeted NO-releasing drug that entered clinical trials. It was synthesized by adding an NO-releasing moiety to ursodeoxycholic acid (UDCA). It is selectively metabolized in hepatocytes to release NO in the liver [4, 19]. Animal studies demonstrated that NCX-1000 could alleviate PHT without changing systemic pressure, but the results of clinical trial were disappointing as it failed to decrease the portal pressure [4, 20-22]. The main cause was that in NCX-1000, the NO-releasing moiety was conjugated with UDCA 24-COOH by ester bonds, whereas free 24-COOH is essential for liver-specific metabolism and the formation of an ester bond at this position might cause the loss of hepatic-targeting effects [23, 24]. A novel liver-targeted NO donor having NO conjugated with UDCA via a threonine spacer (UDCA-Thr-NO, **Figure 1**) was recently developed. In this molecule, NO is linked to the side chain OH group of threonine as a nitrate, which was then attached to bile acid through an amide bond to ensure its plasma stability. The nitrate ester bond can be readily hydrolyzed to release NO. In the meantime, the threonine spacer, which is a natural amino acid and has good biocompatibility, possesses a free α-carboxyl group that can preserve the hepatocyte-specific recognition property of UDCA [25, 26].

The present study was designed to investigate the protective effect of UDCA-Thr-NO against liver injury, such as reduction of PHT in liver cirrhosis, in animal models induced by dimethylnitrosamine (DMN). Moreover, this study was also planned to determine the influences of UDCA-Thr-NO on hepatic function and collagen content, its prevention of cell damage and fibrosis in animal models.

Materials and methods

Materials and animals

UDCA (lot no. 20130105), UDCA-Thr-NO (lot no. 20130328), and compound embryonic bovine liver extract tablets (An-Fa-Te, lot no. 121009) were procured from the Institute of Pharmacology and Toxicology (Beijing, China). DMN and carboxymethylcellulose sodium (CMC) were purchased from Sigma (USA).

Wistar rats (4-week old, weighing 184 ± 9 g) were purchased from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China) and housed in the standard laboratory under controlled environment conditions (23 ± 1°C with 45% ± 5% humidity, and 12 h light/dark cycle) with free access to food and water for 2 weeks before the experiment. The animals and the protocol of this study were approved by the Animal Study Ethics Committee.

Animal study protocol

The cirrhotic rat model was induced by DMN. Male Wistar rats were randomly divided into 7 groups with 15 rats per group: normal control group, model group, UDCA-Thr-NO low-dose, middle-dose and high-dose groups, An-Fa-Te group, and UDCA group. All groups except for the normal control group were injected intraperitoneally with DMN (10 mg/kg/day) three consecutive days per week for 4 weeks. The normal control group was injected with an identical dose of saline at the same site. Then, the UDCA-Thr-NO groups were treated with 15, 30, and 60 mg/kg of UDCA-Thr-NO, respectively, via intragastric administration once a week. The positive control group was given An-Fa-Te at a dose of 28 mg/kg and UDCA at a dose of 60 mg/kg. The normal control and model groups were both treated with 0.5% CMC solution. After 3 weeks of administration, animals

Table 1. Definitions of the liver fibrosis stage (or score)

Stage (score)	Fibrosis	Bridging fibrosis	Bridging fibrosis with nodule formation
Absent (0)	Absent	Absent	Absent
Mild (I)	Mild fibrous expansion (periportal or central)	Absent	Absent
Moderate (II)	Moderate fibrous expansion	Some bridging fibrosis (PP, CC, or PC)	Absent
Marked (III)	Marked fibrous expansion	Marked bridging fibrosis (PP, CC, or PC)	Absent
Extremely marked (IV)	Marked fibrous expansion	Marked bridging fibrosis (PP, CC, or PC)	Present

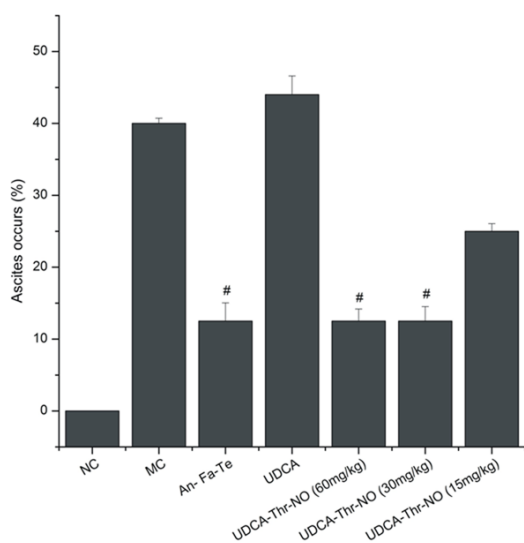CC, Central-central; PC, portal-central; PP, portal-portal.

Figure 2. The impacts of UDCA-Thr-NO, An-Fa-Te, and UDCA on ascites formation in cirrhosis model rats. NC: normal control group, administered 0.5% CMC solution; MC: model control group, administered 0.5% CMC solution. An-Fa-Te: 28 mg/kg of An-Fa-Te; UDCA: 60 mg/kg of UDCA; UDCA-Thr-NO groups: 15, 30 and 60 mg/kg of UDCA-Thr-NO respectively. The results are presented as the means ± SD for 10 animals in each group. $^{*}P < 0.05$ compared with the normal control group; $^{\#}P < 0.05$ compared with the model group.

(five rats per group) were examined for carotid arterial and portal venous pressures. After 4 weeks of administration, the remaining animals (10 rats per group) were anesthetized (ketamine 100 mg/kg and xylazine 20 mg/kg administered intraperitoneally) and blood samples and liver tissues were collected. Rat sera were prepared from whole blood and frozen at -80°C. Livers were rapidly excised, and separate portions were (1) snap-frozen in liquid nitrogen and stored at -80°C and (2) fixed in 10% neutral-buffered formalin. Then, the incidence of ascites was examined and the hepatic fibrosis index was evaluated. The hepatic tissues were also collected for histopathological analysis and hydroxyproline determination by chemical colorimetry.

Liver histopathology

After dehydration, the liver tissue specimens were embedded in paraffin and then cut into 4-μm sections, mounted on glass slides, and baked at 80°C for 1 h. The tissue sections were treated in xylene and rehydrated in an ethanol gradient and finally stained with hematoxylin-eosin (HE) for observing pathological changes in the liver. The hyperplastic state of collagen fibers was observed using the Sirius red (SR) staining method, in which the fibrosis stage was evaluated using a histological scoring scheme adapted from the human Ishak scheme as outlined in **Table 1** [27, 28].

Blood pressure measurement

Carotid artery pressure measurement: Anesthetized rats were fastened to a surgical board, and the left common carotid artery was exposed through a cervical incision. A polyethylene catheter (PE-50) filled with heparin was introduced into the carotid artery via an arteriotomy, and the cannula was connected to a pressure transducer that was linked with a PowerLab PC (A.D. Instruments, MA, USA).

Portal pressure measurement: Anesthetized rats were subjected to laparotomy to expose the main portal vein and separate the superior mesenteric vein. A polyethylene (PE-20) catheter filled with heparin was cannulate to the portal vein, and the pressure was recorded with a pressure transducer as described above.

Serum biochemical assays

The whole-blood samples were centrifuged at 3000 rpm for 10 min to collect the sera. The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl trans-
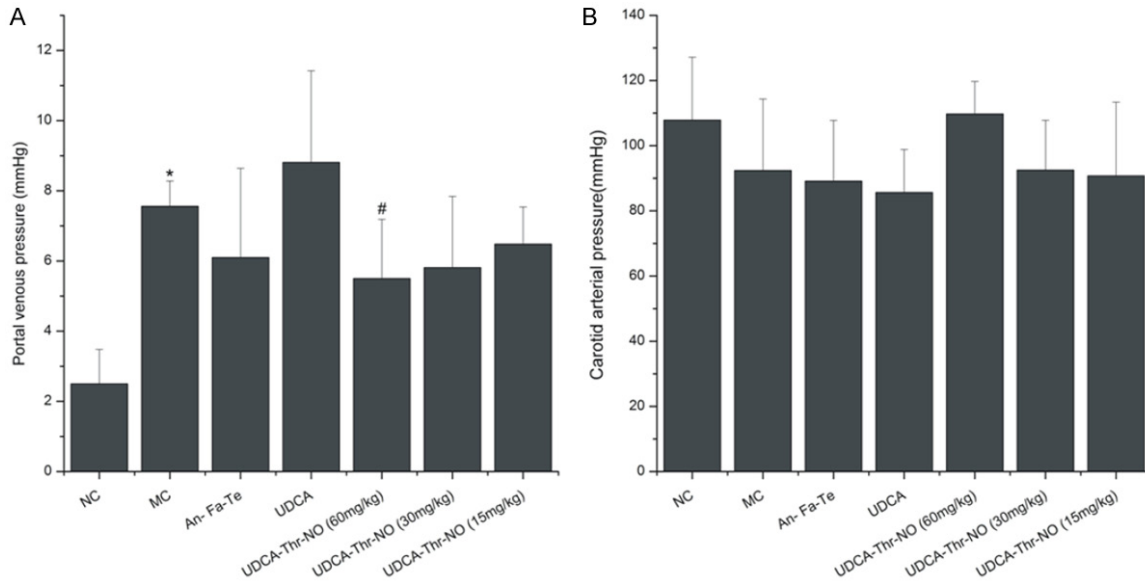
Figure 3. The influence of UDCA-Thr-NO, An-Fa-Te, and UDCA on carotid arterial and portal venous pressures in cirrhosis model rats. NC: normal control group, administered 0.5% CMC solution; MC: model control group, administered 0.5% CMC solution. An-Fa-Te: 28 mg/kg of An-Fa-Te; UDCA: 60 mg/kg of UDCA; UDCA-Thr-NO groups: 15, 30 and 60 mg/kg of UDCA-Thr-NO respectively. The results are presented as the means ± SD for 10 animals in each group. $^*P < 0.05$ compared with the normal control group; $^#P < 0.05$ compared with the model group.

ferase (GGT), albumin (ALB), and total bilirubin (TBiL) levels were measured using the automatic biochemical analyzer (Hitachi 7600, Tokyo, Japan) within 24 h.

Statistical analysis

All of the experimental data were statistically processed using SPSS 19.0 software (SPSS Inc., USA). The values were expressed as mean ± standard deviation (SD). One-way analysis of variance followed by a least significant difference test was used to compare experimental values between groups. A *P* value < 0.05 was considered statistically significant.

Results

Influence of UDCA-Thr-NO on ascites formation in cirrhotic rats

After 4 weeks of intraperitoneal injection of DMN (10 mg/kg/day, 3 days/week), 40% of the rats in the model group rats developed ascites, whereas the rates in the normal control group receiving saline had no sign of ascites formation, suggesting the success in setting up the cirrhotic rat model. It was found that treatments of these rats with high and middle doses of UDCA-Thr-NO and An-Fa-Te significantly reduced the percentage of rats with ascites

from 40% to 12.5% (*P* < 0.05). In contrast, UDCA alone had no significant effect on the percentage of rats that developed ascites (**Figure 2**).

Influence of UDCA-Thr-NO on the blood pressure of cirrhotic rats

Compared with the normal control group rats, a significant increase was found in the portal vein pressure in the DMN model group rats (*P* < 0.05). UDCA-Thr-NO treatment could induce a dose-dependent decrease in the portal vein pressure, and the high-dose UDCA-Thr-NO group was significantly different from that of the model group (5.50 ± 1.69 vs 7.56 ± 0.72, *P* < 0.05). The portal vein pressure was ameliorated in the low-dose and middle-dose UDCA-Thr-NO and An-Fa-Te groups, yet the difference was insignificant. Also, the portal vein pressure was slightly increased in the UDCA group (**Figure 3A**). Besides, no significant difference was noted in carotid arterial pressure between UDCA-Thr-NO-treated groups and the control (**Figure 3B**).

Influence of UDCA-Thr-NO on the serum biochemical indexes of cirrhotic rats

The serum level of TBiL, ALT, AST, and GGT in the DMN-treated model group increased signifi-
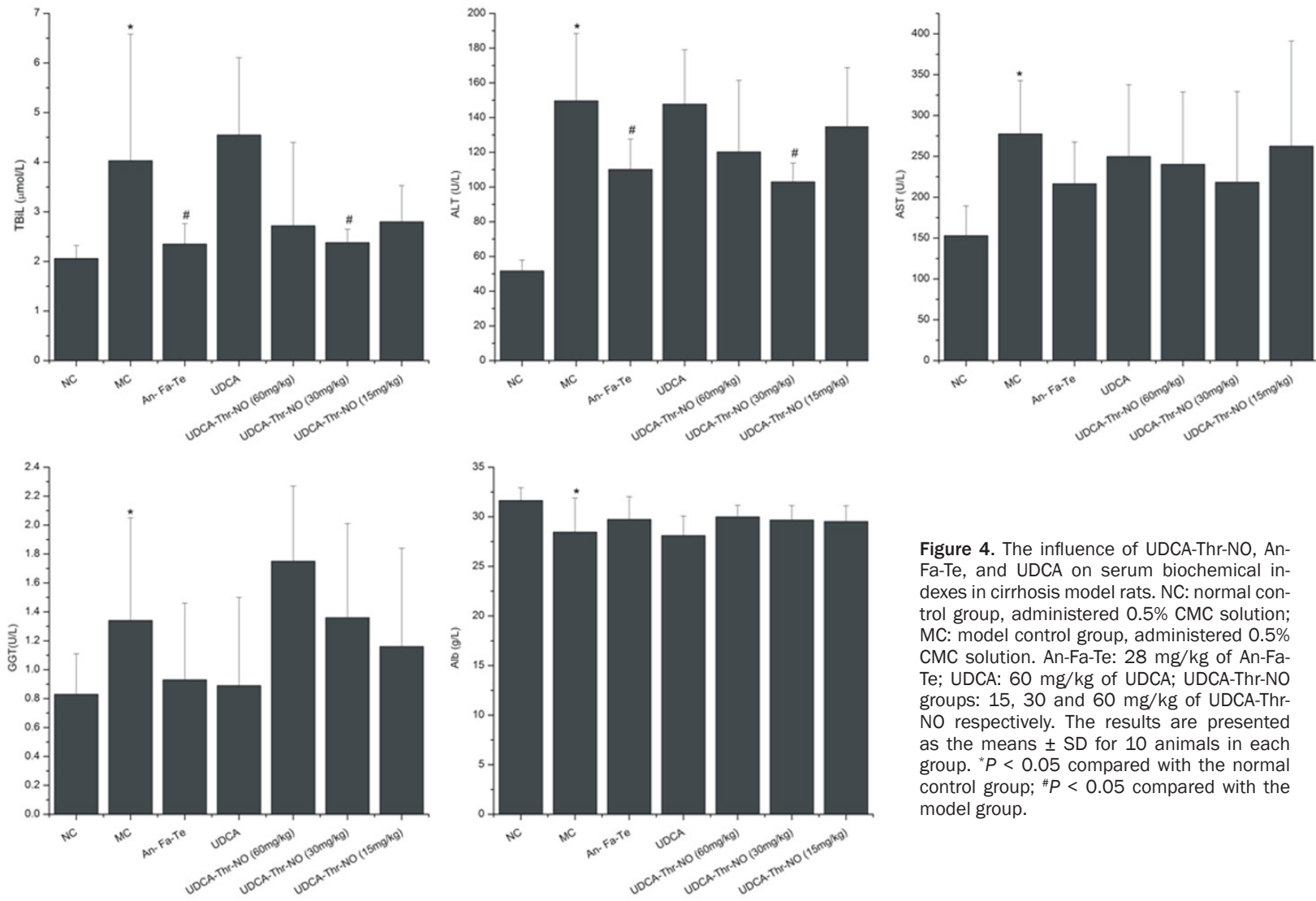
Figure 4. The influence of UDCA-Thr-NO, An-Fa-Te, and UDCA on serum biochemical indexes in cirrhosis model rats. NC: normal control group, administered 0.5% CMC solution; MC: model control group, administered 0.5% CMC solution. An-Fa-Te: 28 mg/kg of An-Fa-Te; UDCA: 60 mg/kg of UDCA; UDCA-Thr-NO groups: 15, 30 and 60 mg/kg of UDCA-Thr-NO respectively. The results are presented as the means ± SD for 10 animals in each group. $^*P < 0.05$ compared with the normal control group; $^{\#}P < 0.05$ compared with the model group.
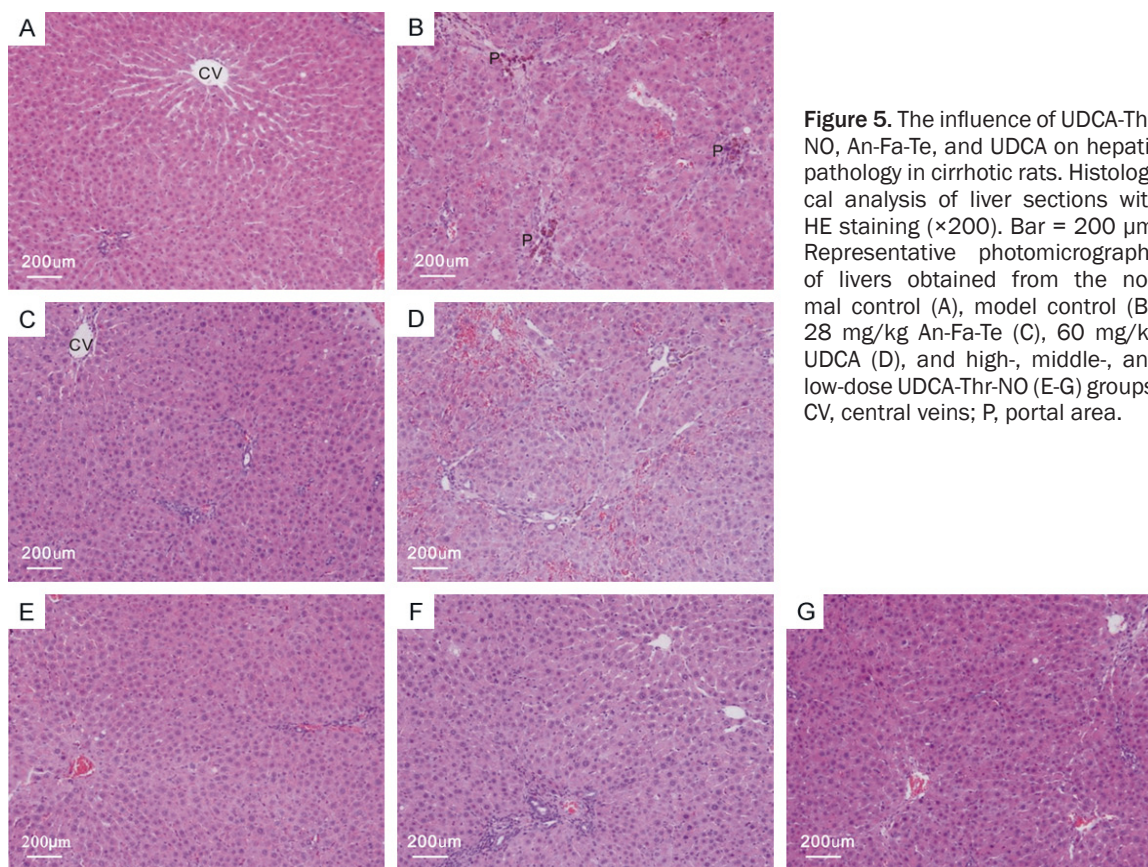
Figure 5. The influence of UDCA-Thr-NO, An-Fa-Te, and UDCA on hepatic pathology in cirrhotic rats. Histological analysis of liver sections with HE staining (×200). Bar = 200 µm. Representative photomicrographs of livers obtained from the normal control (A), model control (B), 28 mg/kg An-Fa-Te (C), 60 mg/kg UDCA (D), and high-, middle-, and low-dose UDCA-Thr-NO (E-G) groups. CV, central veins; P, portal area.
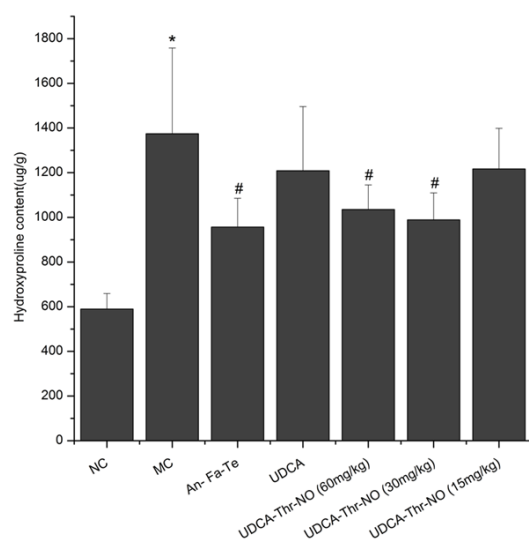

Figure 6. The influence of UDCA-Thr-NO, An-Fa-Te, and UDCA on the hydroxyproline contents in livers of cirrhotic rats. NC: normal control group, administered 0.5% CMC solution; MC: model control group, administered 0.5% CMC solution. An-Fa-Te: 28 mg/kg of An-Fa-Te; UDCA: 60 mg/kg of UDCA; UDCA-Thr-NO groups: 15, 30 and 60 mg/kg of UDCA-Thr-NO respectively. The results are presented as the means ± SD for 10 animals in each group. *$P < 0.05$ compared with the normal control group; #$P < 0.05$ compared with the model group.

cantly ($P < 0.05$) and the level of ALB decreased obviously ($P < 0.05$) as compared to that of the control group (**Figure 4**). Most of the serum indexes showed improvements in both UDCA-Thr-NO and An-Fa-Te groups, while only in middle-dose UDCA-Thr-NO (30 mg/kg) and An-Fa-Te groups that the inhibition of TBiL and ALT levels was statistically significant ($P < 0.05$).

Influence of UDCA-Thr-NO on the liver histopathology of cirrhotic rats

The results of histological analyses performed on HE-stained liver sections (4-µm thick) showed that the hepatic lobule architecture in the normal group was clear, and hepatocyte exhibited a radial pattern distribution from the central vein without denaturation and necrosis. No hepatic sinus stenosis or dilation was observed (**Figure 5A**). On the contrary, the liver of the model group showed expansion of the hepatic sinus stenosis and portal area, and disordered arrangement, swelling, degeneration, and necrosis of the hepatocytes (**Figure 5B**). Compared with the model group, these pathological changes were improved to varying degrees in the UDCA-Thr-NO, An-Fa-Te, and UDCA groups.
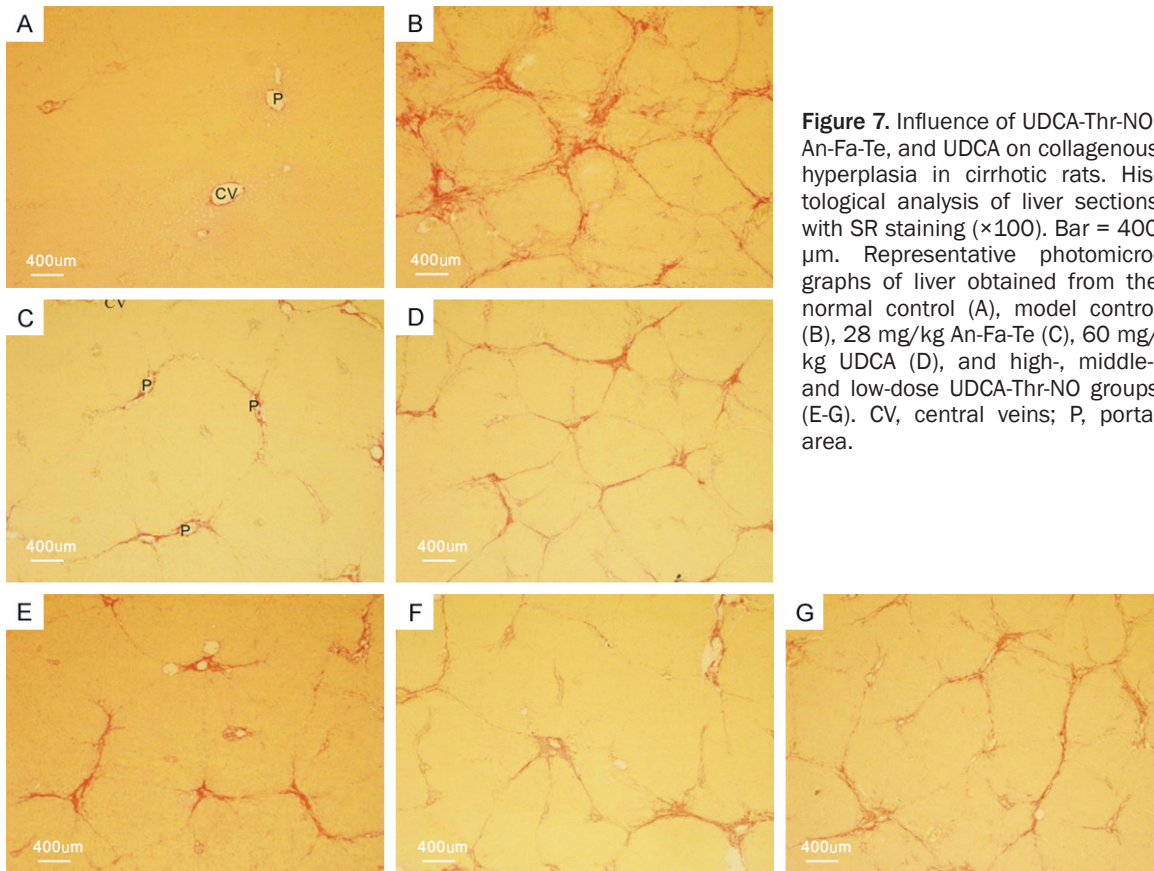
Figure 7. Influence of UDCA-Thr-NO, An-Fa-Te, and UDCA on collagenous hyperplasia in cirrhotic rats. Histological analysis of liver sections with SR staining (×100). Bar = 400 μm. Representative photomicrographs of liver obtained from the normal control (A), model control (B), 28 mg/kg An-Fa-Te (C), 60 mg/kg UDCA (D), and high-, middle-, and low-dose UDCA-Thr-NO groups (E-G). CV, central veins; P, portal area.

Table 2. Liver fibrosis scores for all groups of rats

Group	Dose (mg/kg)	Collagenous hyperplasia grade				
		0	I	II	III	IV
NC	0	10	0	0	0	0
MC	0*	0	0	1	2	6
An-Fa-Te	28#	0	0	4	5	1
UDCA	60	0	0	2	6	1
UDCA-Thr-NO	60#	0	0	6	2	1
	30#	0	0	5	5	0
	15	0	0	3	5	2

NC, normal control group; MC, model control group; each group has 9 or 10 animals. *$P < 0.05$ compared with the normal control group; #$P < 0.05$ compared with the model group.

Also, the improvement was obviously better in the UDCA-Thr-NO and An-Fa-Te groups than in the UDCA group (**Figure 5C-G**).

Influence of UDCA-Thr-NO on the hydroxyproline content in livers of cirrhotic rats

Compared with the normal control group, the level of hydroxyproline in liver tissues of the DNM-treated model group increased significantly ($P < 0.05$) (**Figure 6**). This situation was improved in both UDCA-Thr-NO and An-Fa-Te groups but not in the UDCA group. Moreover, the inhibition on hydroxyproline increase for the high-dose and middle-dose UDCA-Thr-NO and An-Fa-Te groups was statistically significant ($P < 0.05$).

Influence of UDCA-Thr-NO on the liver fibrosis of cirrhotic rats

The effect of UDCA-Thr-NO, An-Fa-Te, and UDCA on DMN-induced cirrhosis in rats was evaluated by histopathological analyses of SR-stained liver sections. Almost no collagen fibers were found in the sections taken from the normal control group, except from the portal area and central vein wall (**Figure 7A**). In the model group, however, the sections showed apparent collagen deposition, and a large number of segmented fibrous septa surrounding the liver tissue and extending toward the hepatic lobule formed a pseudolobule (**Figure 7B**). These pathological changes observed in the model group were ameliorated to varying degrees in the UDCA-Thr-NO and An-Fa-Te groups but not

clearly improved in the UDCA group (**Figure 7C-G**). Moreover, the antifibrotic effect was similar in the high-dose and middle-dose UDCA-Thr-NO and An-Fa-Te groups, which was better than that in the low-dose UDCA-Thr-NO and UDCA groups. The results were in accordance with that of the hydroxyproline content analysis (**Figure 6**).

Influence of UDCA-Thr-NO on the liver fibrosis score of cirrhotic rats

The fibrosis formed in each group of rats was examined and scored according to scales shown in **Table 1**. Clearly, the fibrosis scores listed in **Table 2** indicated an apparent improvement observed in the high-dose and middle-dose UDCA-Thr-NO and An-Fa-Te groups when compared with the model group. Ridit analysis of the results suggested that the differences were statistically significant ($P < 0.05$).

Discussion

PHT is a common liver disease that is closely related to cirrhosis and has a high morbidity and mortality [1]. PHT is usually caused by the increase in intrahepatic resistance and splanchnic blood flow. Thus, reducing the intrahepatic resistance is the principle strategy to treat PHT [2]. As reduced NO production in cirrhosis plays an important role in the hemodynamic abnormalities observed in cirrhosis patients [29], this study was designed to probe the impact of an UDCA-based liver-specific NO donor, UDCA-Thr-NO, on portal hypertensive cirrhotic rats. UDCA and An-Fa-Te were used as the positive controls in this study, as UDCA has antifibrotic effects and is currently used for managing primary biliary cirrhosis and other cholestatic liver diseases [30] and An-Fa-Te is the clinical drug for treating fibrosis and an ancillary drug for steatohepatitis, cirrhosis, and so on [31].

Our results showed that UDCA-Thr-NO could protect against liver injury and improve PHT in the animal model of liver cirrhosis by reducing intrahepatic resistance. The results further supported that UDCA-Thr-NO could modulate both the anatomical and the dynamic components of cirrhosis and PHT. First, UDCA-Thr-NO improved the pathological changes (HE staining) and the serum biochemical indexes such as TBiL and ALT of cirrhotic rats, which could

serve as important indicators of the liver function. The strongest effect was observed with the middle-dose UDCA-Thr-NO (30 mg/kg) group, which was similar to that of An-Fa-Te (28 mg/kg), which was consistent with the results of Li [32]. Second, UDCA, An-Fa-Te, and UDCA-Thr-NO exerted similar antifibrotic effects. Although An-Fa-Te and UDCA-Thr-NO could improve the ascites development at varying degrees, only UDCA-Thr-NO protected against PHT and ascites development induced by DMN at the same time. Also, UDCA-Thr-NO could ameliorate PHT in a dose-dependent manner, most significantly at the higher dose (60 mg/kg), while it had no obvious effect on the carotid arterial pressure. This result might suggest that UDCA-Thr-NO released probably a small amount of NO into the bloodstream to relieve obstruction without significant influence on systemic dynamics. These results were consistent with that obtained from previous studies [4]. Third, the SR staining results showed that the high- and middle-dose UDCA-Thr-NO and An-Fa-Te groups exerted similar antifibrotic effects. Besides, our analysis revealed a statistically significant inhibition of hydroxyproline level, an important index of collagen fiber content, in the high- and middle-dose UDCA-Thr-NO and An-Fa-Te groups, which was in accordance with the pathological results, indicating that UDCA-Thr-NO could ameliorate liver fibrosis in rats with liver cirrhosis.

In conclusion, the liver-specific NO-donor UDCA-Thr-NO was shown to protect against liver injuries via bile acid-mediated transportation and selective release of NO into the liver to reduce the portal pressure and improve PHT. In DMN-induced cirrhosis and PHT rats, UDCA-Thr-NO could effectively ameliorate ascites formation, liver fibrosis, and other pathological features. In addition, compared with the currently available NO donors such as UDCA and NCX-1000, UDCA-Thr-NO was more stable and had better liver-targeting property. UDCA-Thr-NO was also found to release NO to decrease PHT in cirrhotic rats without causing systemic arterial hypotension, which was probably due to its flexibility (all indexes were observed about 20 h after the last dose) to indirectly increase intrahepatic NO bioavailability. Thus, UDCA-Thr-NO is a useful lead compound for the development of new drugs against cirrhosis and its complications, such as PHT.

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