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Original Article

# The effects of proton pump inhibitor on hepatic vascular responsiveness and hemodynamics in cirrhotic rats

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**Abstract**

*Background*: Liver cirrhosis is associated with increased intrahepatic resistance due to hepatic fibrosis and exaggerated vasoconstriction. Recent studies have indicated that proton pump inhibitors (PPIs), in addition to acid suppression, modulate vasoactive substances and vasoresponsiveness. PPIs are frequently prescribed in patients with cirrhosis due to a higher prevalence of peptic ulcers, however other impacts are unknown.

*Methods*: Liver cirrhosis was induced in Sprague−Dawley rats with common bile duct ligation (BDL). On the 29th day after BDL and after hemodynamic measurements, the intrahepatic vascular responsiveness to high concentrations of endothelin-1 (ET-1) was evaluated after preincubation with (1) Krebs solution (vehicle), (2) esomeprazole (30 μM), or (3) esomeprazole plus $N^{\omega}$-nitro L-arginine (NNA, a non-selective NO synthase (NOS) inhibitor, $10^{-4}$ M). After perfusion, the hepatic protein expressions of endothelial NOS (eNOS), inducible NOS (iNOS), cyclooxygenase (COX)-1, COX-2, endothelin-1, DDAH-1 (dimethylarginine dimethylaminohydrolase-1, ADMA inhibitor), DDAH-2, ADMA (asymmetrical dimethyl arginine, NOS inhibitor) were evaluated. In the chronic model, the BDL rats received (1) vehicle; or (2) esomeprazole (3.6 mg/kg/day, oral gavage) from the 1st to 28th day after BDL. On the 29th day and after hemodynamic measurements, plasma liver biochemistry and liver fibrosis were evaluated.

*Results*: Esomeprazole did not affect hepatic ET-1 vasoresponsiveness. The hepatic protein expressions of the aforementioned factors were not significantly different among the groups. There were no significant differences in hemodynamics, liver biochemistry and hepatic fibrosis after chronic esomeprazole administration.

*Conclusion*: PPIs did not affect hepatic vasoresponsiveness or the release of vasoactive substances. Furthermore, they did not influence hemodynamics, liver biochemistry or severity of hepatic fibrosis in the cirrhotic rats.

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*Keywords:* Endothelin; Liver cirrhosis; Nitric oxide; Portal hypertension; Proton pump inhibitor

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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## 1. Introduction

Various kinds of liver damage can lead to fibrogenesis and the formation of regeneration nodules if not controlled appropriately, followed by increased intrahepatic resistance and portal hypertension.[1] In addition to structural changes, increases in vasoconstrictive substances such as endothelin-1 (ET-1) and decreased bioavailability of vasodilatory substances such as nitric oxide (NO) have been shown to play roles in increasing hepatic resistance.[2] Portosystemic collaterals develop to divert stagnant portal blood from a hypertensive portal system, followed by potentially lethal complications. It is widely accepted that modulating intrahepatic resistance is essential to control portal hypertension-related complications.[1]

Proton pump inhibitors (PPIs) exert their potent acid-suppression effect via inhibiting gastric $H^+/K^+$-ATPase in parietal cells. They are frequently used to treat gastrointestinal disorders that involve the production of gastric acid such as peptic ulcer, gastroesophageal reflux disease (GERD), Barrett's esophagus and *Helicobacter pylori* infection.[3–5] The high oral bioavailability of PPIs and their remarkable efficacy in the sustained suppression of gastric acid secretion mean that they are the most popular type of acid suppressant. In addition, the prevalence of gastric ulcer in cirrhotic patients has been reported to be 20.8%, which is significantly higher than the 4.0% reported in healthy controls.[6] A previous study also reported a higher prevalence of reflux esophagitis in Chinese patients with chronic liver diseases.[7] It is therefore reasonable to assume that the use of PPIs in cirrhotic patients is widespread.

The vascular impact of PPIs beyond acid suppression has recently gained increasing attention. For example, lemiprazole has been shown to inhibit contractile responses in isolated rat aortic rings and relax pre-contracted rat aorta.[8] An experimental inhibitor of $H^+/K^+$-ATPase (SCH 28080) has also been shown to relax guinea pig and human pulmonary arteries.[9] In addition, a highly specific inhibitor, NC-1300-B, has been shown to cause profound renal vasodilation and inhibit the release of renin.[10] In contrast, PPIs were shown to result in elevated levels of asymmetric dimethylarginine (ADMA) and reduced levels of NO and endothelium-dependent vasodilation in a murine model and ex vivo human tissues. ADMA is an endogenous inhibitor of NO synthase (NOS). PPIs increase levels of ADMA by inhibiting dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades ADMA.[11] Injections of lansoprazole have been shown to increase levels of ADMA in mice by about 20%.[12] Therefore, it would be interesting to investigate whether PPIs influence the levels of NO, DDAH and ADMA in cirrhosis. A recent study suggested that esomeprazole controlled pulmonary inflammation and fibrosis in a murine model of acute lung injury by suppressing the expressions of pro-inflammatory and fibrogenetic molecules.[13] However, whether PPIs affect liver inflammation and fibrosis has yet to be investigated.

Considering the vascular actions of PPIs beyond acid suppression and the frequent prescription of PPIs in cirrhotic patients, this study aimed to investigate hemodynamic changes, intrahepatic vascular responsiveness to ET-1, liver biochemistry and fibrosis in cirrhotic rats exposed to PPIs.

## 2. Methods

### 2.1. Animal model

Male Sprague–Dawley rats weighing 240–270 g at the time of surgery were used for the experiments. The rats were housed in plastic cages and allowed free access to food and water. All rats were fasted for 12 h before the operation. Secondary biliary cirrhosis was induced using common bile duct ligation (BDL).[14] Under ketamine anesthesia (100 mg/kg, intramuscularly), the common bile duct was doubly ligated with 3-0 silk. The first ligature was made below the junction of the hepatic ducts and the second ligature was made above the entrance of the pancreatic duct, followed by sectioning the common bile duct between the ligatures. A high yieldof secondary biliary cirrhosis was noted 4 weeks after the ligation.[15] To avoid coagulation defects, the BDL rats received weekly vitamin K injections (50 μg/kg intramuscularly).[16] This study was approved by Taipei Veterans General Hospital Animal Committee (IACUC 2015-109). All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

### 2.2. Measurement of systemic and portal hemodynamics

The right carotid artery was cannulated with a PE-50 catheter that was connected to a Spectramed DTX transducer (Spectramed Inc., Oxnard, CA, USA). Continuous recordings of mean arterial pressure (MAP), heart rate (HR), and portal pressure (PP) were performed on a multi-channel recorder (model RS 3400, Gould Inc., Cupertino, CA, USA). The external zero reference was placed at the level of the midportion of each rat. The abdomen was then opened with a midline incision, and the mesenteric vein was cannulated with a PE-50 catheter connected to a Spectramed DTX transducer. The abdominal cavity was closed and the PP was recorded on the Gould model RS 3400 recorder.[17]

The superior mesenteric artery (SMA) was identified at its aortic origin, and a 5-mm segment was gently dissected free from surrounding tissues. A pulsed-Doppler flow transducer (T206 small animal blood flow meter, Transonic System Inc., Ithaca, NY, USA) was then used to measure SMA flow.[18] Portal flow was also measured using the flow transducer. The measurement point was as proximal to the liver as possible.

Cardiac output (CO) was measured by thermodilution, as previously described.[19] Briefly, a thermistor was placed in the aortic arch just distal to the aortic valve and a thermal indicator (100 μL of normal saline) was injected into the right

atrium through a PE-50 catheter. The aortic thermistor was connected to a Columbus Instruments Cardiotherm 500-AC-R (Columbus Instruments International Co., OH, USA). Five thermodilution curves were obtained for each cardiac output measurement. The final value was obtained from the arithmetic mean of the computer results. Cardiac index (CI, ml/min/100 g body weight (BW)) was calculated as CO per 100 g BW. Systemic vascular resistance (SVR, mmHg/ml/min/100 g BW) was calculated by dividing the MAP by the CI. SMA resistance (mmHg/ml/min/100 g BW) was calculated as (MAP-PP)/SMA flow per 100 g BW. Portal resistance (mmHg/ml/min/100 g BW) was calculated as PP/portal flow per 100 g BW.

### 2.3. In situ perfusion of the liver

*In situ* perfusion was performed as previously described with several modifications.[20] Briefly, both jugular veins were cannulated with 16-gauge Teflon cannulas to ensure an adequate outflow without any resistance. Heparin (200 U/100 g BW) was injected through one of the cannulas. The abdomen was then opened and a 16-gauge Teflon cannula was inserted into the portal vein, followed by ligation of the hepatic artery. To exclude the collateral from perfusion, a second loose ligature around the distal portal vein was tied. Open circuit perfusion was then started with perfusion of oxygenated Krebs solution in a warm chamber. Both jugular vein cannulas were simultaneously opened to allow for complete washout of the blood. All of the experiments were performed 15 min after starting perfusion at a constant rate of 40 ml/min. In each individual preparation, after testing the experimental agents, the contracting capability of the intrahepatic vascular bed was challenged with a 125-mM potassium chloride solution. The criteria for liver viability included gross appearance and stability of perfusion pressure.

### 2.4. Western analysis

Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until required. Protein extracts were obtained by pulverization in a grinder with liquid nitrogen, followed by the addition of 1 ml of lysis buffer (phosphate-buffered solution containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)), and 0.05% protease inhibitor cocktail solution (Roche Diagnostics GmbH, Penzberg, Germany) for each 100 mg powdered sample. The protein concentration was determined for each sample by the Bradford method.[21] An aliquot of 20–40 μg protein from each sample was dissolved in sample buffer (63 mmol/l of Tris–HCl, pH 6.8, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.005% bromophenol blue), and 10 μg of positive control was separated on denaturing SDS-10% polyacrylamide gels by electrophoresis (Mini-PROTEAN® 3 Cell, Bio-Rad Laboratories, Hercules, CA, USA). Pre-stained protein markers (SDS-PAGE Standards, Bio-Rad Laboratories, Hercules, CA, USA) were used to determine molecular weights. Proteins were then transferred to a polyvinylidene difluoride membrane (Immum-Blot™ PVDF Membrane, Bio-Rad Laboratories, Hercules, CA, USA) using a semi-dry electroblotting system (Trans-Blot® SD Semi-dry Electrophoretic Transfer Cell, Bio-Rad Laboratories, Hercules, CA, USA) for 1.5 h at 4 °C. To block non-specific binding, the membranes were blocked for 30 min with 3% non-fat dry milk in TBS-T, pH 7.4 (25 mmol/l Tris base-137 mmol/l NaCl-2.7 mmol/l KCL-1% Tween 20). Blots were incubated with the primary antibody, diluted with 3% non-fat dry milk in TBS-T for 90 min at room temperature and washed. The blots were then incubated for 90 min with the secondary antibody and washed. Specific proteins were detected by enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, Merck Millipore Co., Billerica, MA, USA). A computer-assisted video densitometer and digital system (BioSpectrum® 600 Imaging System, Ultra-Violet Products Ltd., Upland, CA, USA) were used to scan and photograph the blots, and then the signal intensity (integral volume) of the appropriate band was analyzed.

### 2.5. Measurements of plasma levels of the biochemistry parameters

Plasma levels of aspartate transaminase (AST), alanine transaminase (ALT) and total bilirubin were determined using VITROS DT60 II and DTSC II analyzers (Ortho-Clinical Diagnostic Inc., NJ, USA).

### 2.6. Hepatic fibrosis determination with Sirius red staining

Liver paraffin sections were stained using a Sirius red staining kit (Polysciences Inc., Warrington, PA, USA). Image J software was used to measure the percentage of Sirius red-stained areas. Briefly, a grayscale image was used, and the red-stained collagen was isolated using the thresholding function. The thresholded area was then measured as the percentage of the thresholded area per image.[18]

### 2.7. Hematoxylin and eosin staining

Livers were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E).

### 2.8. Statistical analysis

All results are expressed as mean ± S.E.M. Statistical analyses were performed using an independent t-test or one-way ANOVA as appropriate. SPSS version 21 software for Windows (SPSS Inc., Chicago, IL, USA) was used for all analyses. Tukey HSD was used for the post-hoc test. The comparisons between different sets of data were considered statistically significant at two-tailed $p$-values of less than 0.05.

### 2.9. Study protocol

Liver cirrhosis was induced by BDL in male Sprague–Dawley rats.

#### 2.9.1. Acute study

The hepatic vascular responsiveness to high concentrations of ET-1 ($10^{-10}$, $10^{-9}$, $3 \times 10^{-9}$, $10^{-8}$, and $3 \times 10^{-8}$ M) in response to one of the following preincubation conditions for 1 h was evaluated in the BDL rats in an *in situ* liver perfusion model: (1) vehicle (Krebs solution); (2) Esomeprazole (30 μM); (3) Esomeprazole plus $N^{\omega}$-nitro L-arginine (NNA, $10^{-4}$ M). After the perfusion experiments, the livers of these groups were dissected for Western blotting of eNOS, iNOS, cyclooxygenase (COX)-1, COX-2, endothelin-1, DDAH-1, DDAH-2, and ADMA.

#### 2.9.2. Chronic study

The BDL rats were given (1) vehicle (DW, distilled water) or (2) esomeprazole (3.6 mg/kg/day, oral gavage) from the 1st to 28th day after BDL. On the 29th day, after portal and systemic hemodynamic measurements (MAP, HR, PP, CO, SMA flow, PV flow), blood was withdrawn to determine plasma liver biochemistry data, and the livers were dissected for H&E and Sirius red staining to evaluate the extent of liver fibrosis.

## 3. Results

### 3.1. The effects of preincubation with PPI (esomeprazole) on hepatic vascular responsiveness to vasoconstrictor in the cirrhotic rats

Table 1 shows that the baseline hemodynamic parameters evaluated before the perfusion study were not significantly different among the three groups. Fig. 1 depicts the hepatic concentration–response curves to ET-1. The baseline perfusion pressure was not significantly different among the groups (perfusion pressure (mmHg): vehicle (V, n = 6) vs. esomeprazole (E, n = 7) vs. esomeprazoe + NNA (E + N, n = 5): 11.6 ± 0.6 vs. 11.4 ± 0.5 vs. 10.5 ± 0.5, respectively, $p > 0.05$ among groups). There were no significant differences between the groups preincubated with the vehicle or esomeprazole ($p > 0.05$ at all concentrations of ET-1), whereas the addition of NNA to esomeprazole significantly increased changes in the perfusion pressure compared with the vehicle or esomeprazole at an ET-1 concentration of $10^{-10}$ (perfusion pressure change (mmHg): V vs. E vs. E + N: 21.0 ± 2.2 vs. 20.5 ± 2.8 vs. 33.4 ± 5.0, V vs. E + N: $p = 0.024$, E vs. E + N: $p = 0.017$), $10^{-9}$ M (V vs. E vs. E + N: 25.6 ± 2.5 vs. 25.3 ± 2.5 vs. 37.7 ± 5.4, V vs. E + N: $p = 0.026$, E vs. E + N: $p = 0.020$), $3 \times 10^{-9}$ M (V vs. E vs. E + N: 28.6 ± 3.4 vs. 30.9 ± 2.3 vs. 40.9 ± 4.3, V vs. E + N: $p = 0.021$, E vs. E + N: $p = 0.047$), and $10^{-8}$ M (V vs. E vs. E + N: 34.7 ± 4.1 vs. 34.3 ± 1.6 vs. 46.4 ± 4.1, V vs. E + N: $p = 0.027$, E vs. E + N: $p = 0.020$), respectively.

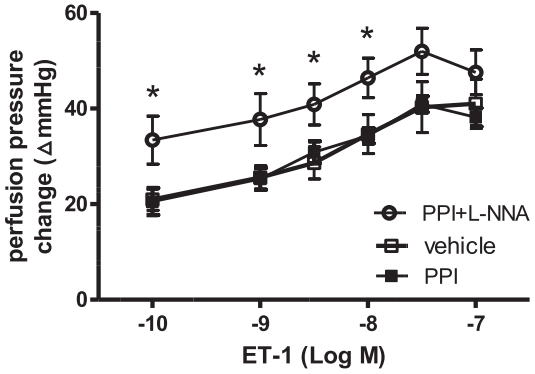


Fig. 1. The effects of different preincubation conditions on hepatic vascular responsiveness to ET-1. There were no significant differences between the vehicle- and esomeprazole-preincubated groups. The addition of L-NNA to esomeprazole significantly increased changes in the perfusion pressure at ET-1 concentrations of $10^{-10}$, $3 \times 10^{-10}$, $10^{-9}$, $3 \times 10^{-9}$ and $10^{-8}$ M, (*$p < 0.05$).

### 3.2. The effects of PPI (esomeprazole) on hepatic vasoactive factors and protein expressions

Fig. 2 shows the protein expressions of the hepatic vasoactive substances iNOS, eNOS, COX1, COX2, ET-1, ADMA,

Table 1
Baseline body weight and hemodynamic parameters of the cirrhotic rats before the perfusion experiments.

| | vehicle (n = 6, v)<br>*p*: v vs. e | esomeprazole (n = 7, e)<br>*p*: e vs. eN | esomeprazole + NNA (n = 5, eN)<br>*p*: eN vs. v |
|---|---|---|---|
| **BW (g)** | 353 ± 15 | 388 ± 16 | 379 ± 7 |
| ***p*** | 0.199 | 0.632 | 0.412 |
| **MAP (mmHg)** | 117 ± 5 | 134 ± 6 | 134 ± 5 |
| ***p*** | 0.062 | 0.982 | 0.067 |
| **HR (beats/min)** | 360 ± 12 | 395 ± 14 | 372 ± 15 |
| ***p*** | 0.150 | 0.242 | 0.742 |
| **PP (mmHg)** | 18.7 ± 1.1 | 19.1 ± 0.7 | 20.1 ± 1.3 |
| ***p*** | 0.700 | 0.480 | 0.307 |

PPI = proton pump inhibitor; NNA=$N^{\omega}$-nitro-L-arginine; BW = body weight; MAP = mean arterial pressure; HR = heart rate; PP = portal pressure.

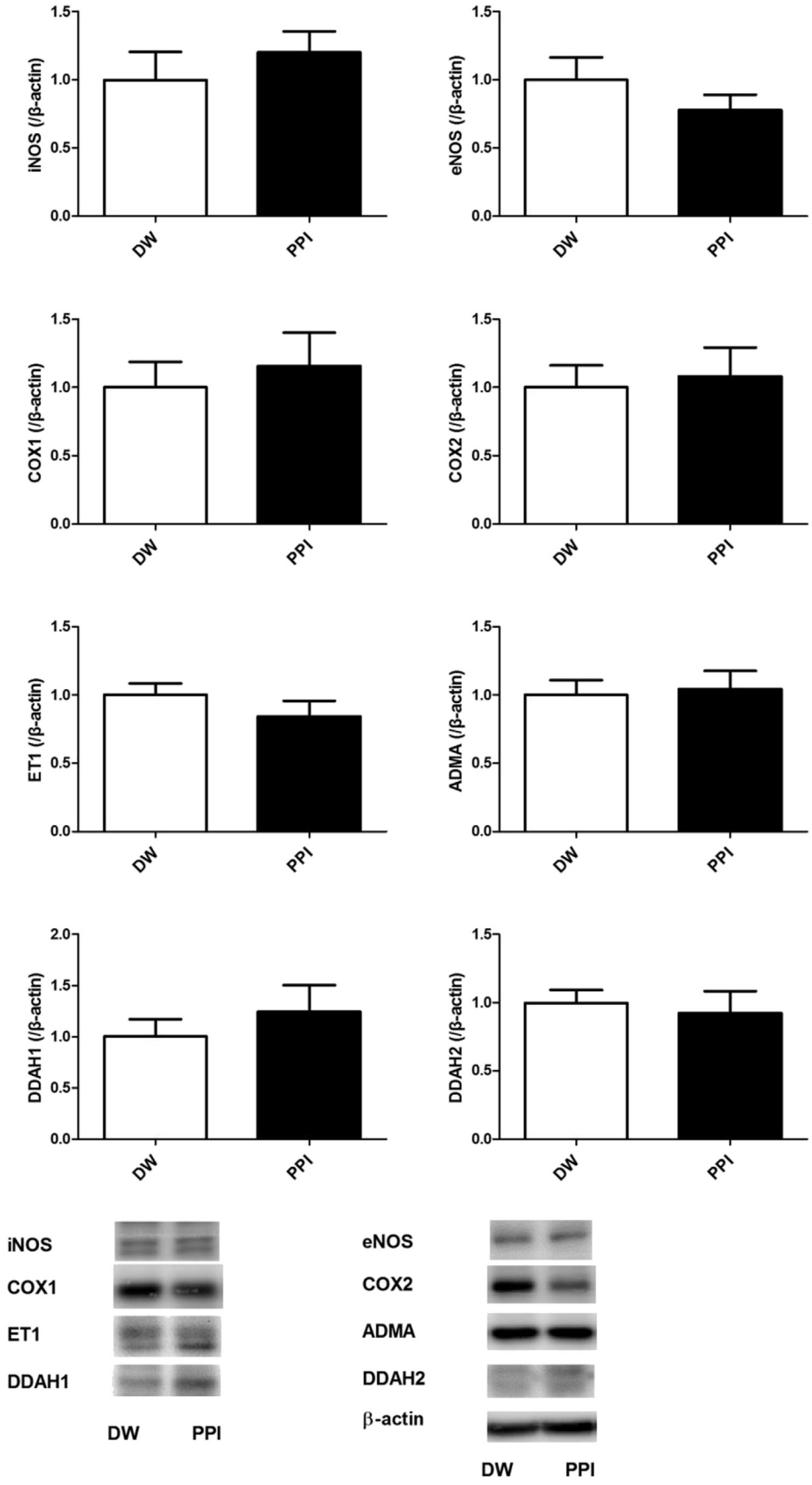


Fig. 2. The effects of esomeprazole on the protein expressions of hepatic vasoactive substances. The protein expressions of iNOS, eNOS, COX1, COX2, ET-1, ADMA, DDAH-1 and DDAH-2 were not significantly different between the vehicle (distilled water, DW)- and esomeprazole-treated groups (all $p > 0.05$).

Table 2
Body weight and hemodynamic parameters of the cirrhotic rats with chronic vehicle (distilled water) or esomeprazole treatment.

| | vehicle (n = 7) | esomeprazole (n = 7) | *p* |
|---|---|---|---|
| **BW (g)** | 407 ± 9 | 381 ± 9 | 0.058 |
| **MAP (mmHg)** | 121 ± 3 | 119 ± 7 | 0.774 |
| **HR (beats/min)** | 322 ± 9 | 314 ± 17 | 0.674 |
| **PP (mmHg)** | 17.6 ± 1.0 | 16.7 ± 1.1 | 0.591 |
| **CI (ml/min/100 g)** | 41.9 ± 2.6 | 37.4 ± 2.8 | 0.259 |
| **SVR (mmHg/ml/min/100 g)** | 3.0 ± 0.2 | 3.2 ± 0.2 | 0.882 |
| **SMA flow (ml/min/100 g)** | 7.5 ± 0.4 | 7.7 ± 0.8 | 0.835 |
| **SMAR (mmHg/ml/min/100 g)** | 14.0 ± 1.0 | 14.0 ± 1.5 | 0.884 |
| **Portal flow (ml/min/100 g)** | 8.3 ± 0.9 | 7.8 ± 0.6 | 0.345 |
| **PVR (mmHg/ml/min/100 g)** | 2.2 ± 0.2 | 2.2 ± 0.1 | 0.376 |

BW = body weight; MAP = mean arterial pressure; HR = heart rate; PP = portal pressure; CI = cardiac index; SVR = systemic vascular resistance; SMA = superior mesenteric artery; SMAR=SMA resistance; PVR = portal vein resistance.

DDAH-1 and DDAH-2. There were no significant differences in the protein expressions between the vehicle- and esomeprazole-treated groups.

### *3.3. The effects of PPI (esomeprazole) on portal hypertension-related hemodynamic derangements*

Table 2 shows the effects of esomeprazole on the portal hypertension-related hemodynamic parameters including BW, MAP, HR, PP, CI, SVR, SMA flow, SMA resistance, PV flow and PV resistance. There were no significantly differences between the vehicle- and esomeprazole-treated groups (all $p > 0.05$).

### *3.4. Plasma liver biochemistry parameters*

The plasma liver biochemistry parameters are shown in Table 3. Chronic esomeprazole treatment did not affect ALT, AST and total bilirubin levels.

### *3.5. The effects of PPI (esomeprazole) on hepatic fibrosis*

Fig. 3 reveals the severity of hepatic fibrosis as evaluated by Sirius red staining. There was no significant difference between the two groups. H&E staining showed a similar extent of liver fibrosis in the vehicle- and esomeprazole-treated groups.

## 4. Discussion

In this study, we evaluated the effects of PPI (esomeprazole) on the hepatic system of cirrhotic rats. The results showed that esomeprazole did not affect vascular constriction, portal and systemic hemodynamics, or hepatic fibrosis. Furthermore, the protein expressions of hepatic vasoactive substances were not affected by chronic esomeprazole treatment.

Table 3
Liver biochemistry data of the cirrhotic rats with vehicle or esomeprazole treatment.

| | vehicle (n = 7) | esomeprazole (n = 6) | *p* |
|---|---|---|---|
| **AST (U/L)** | 480 ± 87 | 505 ± 96 | 0.950 |
| **ALT (U/L)** | 116 ± 16 | 135 ± 32 | 0.601 |
| **Total bilirubin (mg/dl)** | 9.3 ± 1.6 | 8.2 ± 0.9 | 0.592 |

AST = aspartate transaminase; ALT = alanine transaminase.

The vascular actions of PPIs are controversial. Lansoprazole has been reported to increase the production of NO in the corpus mucosa and increase mucosal blood flow.[22] NOS inhibitors and removal of vascular endothelium have been shown to partially inhibit omeprazole- and leminoprazole-induced relaxation of rat aortic rings.[8] Another study reported that omeprazole increased the gastric mucosal expression of endothelial NOS (eNOS) by 68.7% and ET-1 by 12.2% in an animal model with indomethacin-induced gastric mucosal injury.[23] However, other studies have reported conflicting results, such as omeprazole reducing the expression of NO generated by human saphenous vein segments. In addition, PPIs have been reported to impair endothelium-dependent vasodilation in isolated murine vessels.[11] The difference in results may be due to different disease models, experimental settings, and administered agents. In this study, the specific effect of a PPI on hepatic vascular tone in cirrhotic rats was evaluated by *in situ* liver perfusion. The concentration–response curve to ET-1 was not affected by preincubation with esomeprazole, indicating that the PPI did not influence vascular contractility of the liver. In addition, the hepatic protein expressions of iNOS, eNOS, ADMA, DDAH-1 and DDAH-2 were not significantly different between the vehicle- and esomeprazole-treated cirrhotic rats, which is compatible with the findings of perfusion experiments.

The hemodynamic effects of PPIs vary among studies. A recent study in diabetic patients indicated that PPIs increase blood pressure,[24] and that the reduced vascular bioavailability of NO may play a role.[11] In contrast, other studies have suggested that PPIs reduce blood pressure.[25,26] The results of the current study indicate that esomeprazole did not influence the hemodynamics in the cirrhotic rats. Nevertheless, further investigations are required to verify our findings.

In conclusion, esomeprazole did not affect the hepatic vascular responsiveness to ET-1 or the protein expressions of hepatic vasoactive factors. Furthermore, chronic esomeprazole administration did not influence portal hypertension-related hemodynamic parameters or the severity of hepatic fibrosis, suggesting that the use of PPIs in cirrhotic patients may not be harmful in terms of these aspects, although further clinical surveys are required to verify our findings.

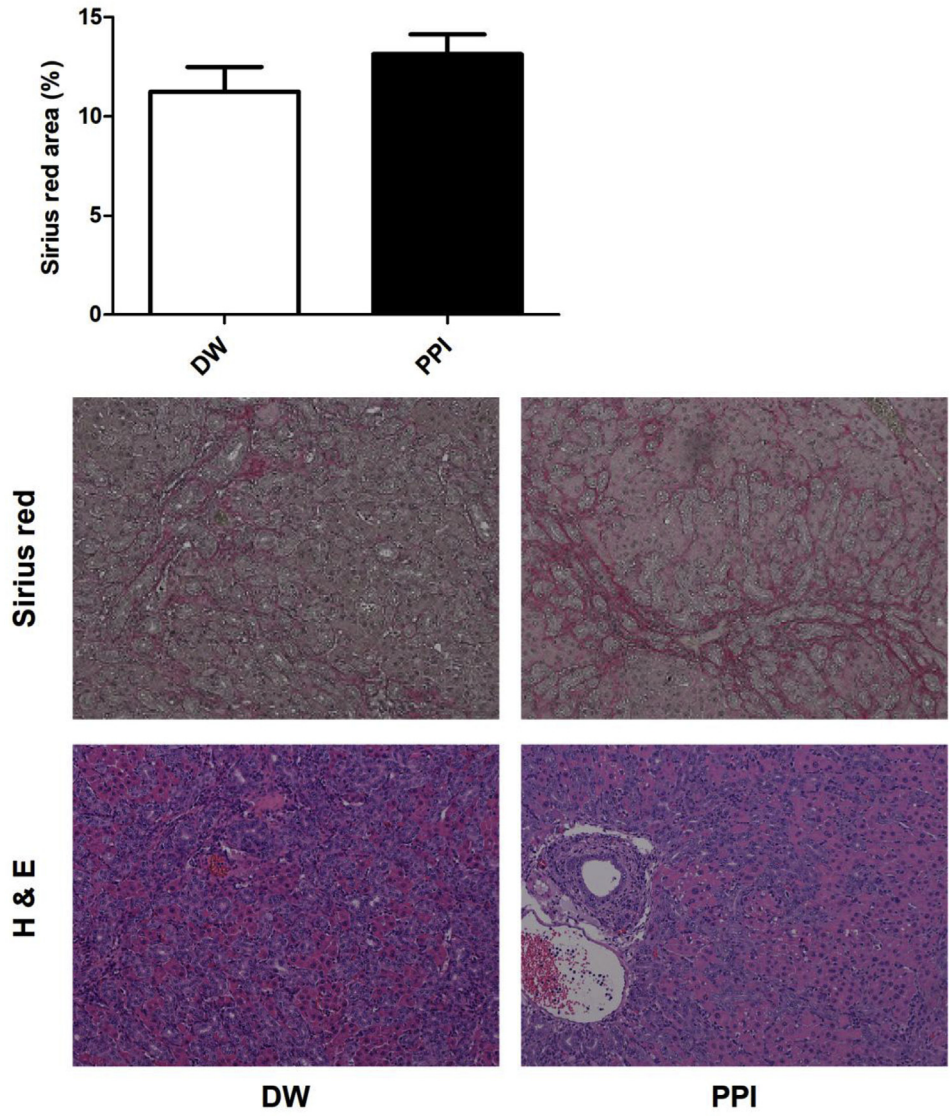


Fig. 3. The effects of esomeprazole on hepatic fibrosis. The severity of hepatic fibrosis was evaluated by Sirius red staining. There was no significant difference between the vehicle (distilled water, DW)- and esomeprazole-treated groups ($p > 0.05$). The middle panel shows representative figures of Sirius red staining (magnification, 40×). The lower panel shows representative figures of H&E staining (magnification, 40×).

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