

Original Article

Hydrogen sulfide ameliorates renal function in cirrhotic rats

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Abstract: Objective: To investigate the role of hydrogen sulfide (H₂S)/cystathionine-γ-lyase (CSE) system in the pathogenesis of experimental hepatorenal syndrome. Methods: Rats were divided into sham group, cirrhotic group and NaHS-treated group. Biliary cirrhosis was established by bile duct ligation in the last two groups. Rats in the NaHS-treated group received an intraperitoneal injection of NaHS (56 μmol/kg/day) for the last 14 days before sample collection. The expression of renal CSE mRNA was measured by real time quantitative PCR, while protein expression was determined by immunohistochemical analysis. Hematoxylin and eosin staining was performed to observe liver cirrhosis and renal structure. Renal artery blood flow, mean arterial pressure and portal vein pressure, 24-hour total urinary volume, serum and urine sodium concentrations, and creatinine clearance rate (Ccr) were also measured. Results: Compared with the sham group, the cirrhotic group demonstrated decreased renal CSE mRNA and protein expression, levels of H₂S in renal arterial blood, 24-hour total urinary volume, renal artery blood flow, serum and urine sodium concentration, and a significant decrease in the Ccr ($P < 0.05$). Compared with the cirrhotic group, NaHS-treated group increased 24-hour total urinary volume, renal artery blood flow, serum sodium concentration, Ccr, and renal CSE mRNA and protein expression ($P < 0.05$). Conclusion: Renal dysfunction in cirrhotic rats is associated with and, in part, mediated by decreased H₂S production and renal CSE expression, and exogenous H₂S can ameliorate renal function in cirrhotic rats.

Keywords: Hydrogen sulfide, cystathionine-γ-lyase, hepatorenal syndrome, bile duct ligation, biliary cirrhosis

Introduction

Renal dysfunction is common in clinic for patients with advanced liver disease or cirrhosis, its severity varies from electrolyte-balance disturbances and water retention to hepatorenal syndrome (HRS) [1]. HRS is regarded as a functional renal failure since there is no structural damage in renal histology, and it could be reversed in many cases [2]. However, it could increase the risk of mortality in these patients [3, 4].

It has been widely believed that marked renal vasoconstriction in the presence of splanchnic and systemic vasodilation may play an important role in the pathogenesis of HRS [5-7]. Carbon monoxide (CO) is a potent vasodilator, it can activate soluble guanylate cyclase and relax vascular smooth muscle in a cGMP-

dependent or cGMP-independent manner [8, 9]. Our previous studies have shown that CO, a byproduct of heme oxygenase-1 (HO-1), was less produced in kidneys of liver cirrhotic rats, and we drew the conclusion that decreased renal heme oxygenase-1 (HO-1)/CO expression is an important contributor to the development of experimental HRS [10].

Recently, hydrogen sulfide (H₂S) has been regarded as the third endogenous signaling gaso-transmitter in addition to nitric oxide (NO) and CO, and has been shown to play important roles in both normal physiological conditions and in the process/progress of several diseases [11], including vasorelaxation by activating the K_{ATP} channel, a different way from NO and CO [12, 13]. H₂S is endogenous produced by two key enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), and CSE is the

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major contributor in kidney [14]. Since the characteristics of H₂S are familiar to CO and NO, it is intuitive that H₂S/CSE system may also be involved in the development of HRS. In this study, we aimed to clarify the change of H₂S/CSE system in kidneys and the effects of H₂S on renal function in cirrhotic rats.

Materials and methods

Animals care

The experimental protocols were approved by the Animal Care and Use Committee of Dalian Medical University, in accordance with the guidelines established by the Canadian Council on Animal Care.

Reagents

The reagents used in the present study were: Sodium hydrogen sulfide (NaHS) (Sigma-Aldrich, St Louis, MO, USA) was freshly dissolved with 0.9% saline. TRIzol Reagent, (KGA1203, Nanjing KeyGEN Biotech. CO.,LTD. Nanjing, China), PrimeScript[®] RT Master Mix Perfect Real Time (TaKaRa Code:DRR036A, TaKaRa Biotechnology, Dalian, China), SYBR[®] Premix Ex Taq[™], (TaKaRa Code:DRR420A), rabbit anti-mouse CSE antibody (Boster Biological Technology, Wuhan, China), anti-rabbit IgG (MaxVision[™]2, MaiXin Biotechnology, Fuzhou, China), TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa).

Animal model and grouping

Healthy male Sprague Dawley rats, weighing 200-220 g, were gained from the laboratory Animal Center of Dalian Medical University. The rats were randomly divided into a sham group ($n = 8$), a cirrhotic group ($n = 12$) and a NaHS-treated group ($n = 12$). The rats were well cared for 3 days before any experimental protocols. Biliary cirrhosis was established by modified bile duct ligation (BDL) method introduced before [15] in rats in the cirrhotic group and NaHS-treated groups. The rats were continuously well fed and housed for a further 4 weeks after surgery, and then we collected the samples. The sham group also underwent laparotomy, and the bile duct was isolated, but was not ligated. Rats in the NaHS-treated groups received an intraperitoneal injection of NaHS (56 $\mu\text{mol/kg/day}$) for the last 14 days [16].

After the establishment of animal models, the number of rats was reduced to 10 in NaHS-treated group and 8 in cirrhotic group. The three groups of rats were housed in metabolic cages for the last 24 hours and urine was collected to measure volume and sodium and creatinine (Cre) levels.

Sample collection

Four weeks after surgery, the rats were anesthetized with ether. Renal artery blood flow was measured by ultrasound (LOGIQ7, GE, USA). Mean arterial pressure (MAP) and portal vein pressure (PVP) were measured by pressure transducers (BL-420F biological experimental system, Chengdu Technology & Market Co. Ltd., China). Then 1 mL of renal arterial blood was collected to measure H₂S. Serum levels of bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), Cre, sodium as well as urine Cre and urine sodium concentrations were measured by using a Hitachi 7600-110 automatic biochemical analyzer (Hitachi Co., Tokyo, Japan). The Cre clearance rate (Ccr) was calculated as urine Cre \times urine volume/serum Cre. The left kidney and one liver lobe were excised and part of the tissues were fixed in 10% neutral formalin solution and embedded in paraffin, while the remaining tissues were preserved at -80°C for PCR.

Measurement of H₂S in renal artery

A sample of plasma (75 μL) was mixed with 100 μL of distilled water and 300 μL of 10% trichloroacetic acid. Then, 150 μL of 1% zinc acetate was added to Eppendorf tubes, along with N,N-dimethyl-p-phenylenediamine sulfate (20 μM) in 7.2 M HCl and FeCl₃ (30 μM ; 133 μL) in 1.2 M HCl were added to the reaction mixture. After 20 minutes of incubation at room temperature, the absorbance of the solution was measured at a wavelength of 670 nm with a spectrometer (UV-2550, Shimadzu, Japan). All samples were assayed in duplicate, and H₂S concentration was calculated against a calibration curve of the standard NaHS solution.

Real time quantitative PCR

Quantification of the expression level of renal CSE mRNA was performed using real time qPCR. Total RNA was extracted from rat kidneys using TRIzol reagent (Nanjing KeyGen

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Table 1. Comparison of serum AST, ALT, H₂S Cre, Ccr, sodium and urine Cre, sodium

	Sham group	Cirrhosis group	NaSH-treated group
Serum Cre (μmol/L)	29.7 ± 1.79	37.3 ± 6.35 ^a	32.9 ± 5.74 ^b
Urine Cre (μmol/L)	7.14 ± 1.13	8.15 ± 2.46	6.29 ± 1.26
Serum sodium (mmol/L)	142.74 ± 3.41	138.69 ± 2.86 ^a	140.46 ± 3.14 ^b
Urine sodium (mmol/L)	90.62 ± 10.17	73.14 ± 8.92 ^a	82.33 ± 8.75 ^b
Ccr (mL/min)	0.22 ± 0.03	0.13 ± 0.05 ^a	0.17 ± 0.04
AST (IU/L)	146.9 ± 38.43	204.6 ± 54.48 ^a	188.4 ± 44.37 ^b
ALT (IU/L)	34.36 ± 5.41	87.37 ± 7.82 ^a	44.49 ± 5.83 ^b
H ₂ S (umol/L)	370.46 ± 52.37	143.77 ± 39.64 ^a	217.76 ± 34.68 ^b

Values are expressed as means ± SD (*n* = 8-10 per group). ^a*P* < 0.05 vs Sham group; ^b*P* < 0.05 vs Cirrhotic group. Cre: creatinine; Ccr: creatinine clearance rate; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

Biotech. Co., Ltd.). Then reverse transcription was performed by using random primers provided with the Takara PCR kit, following the manufacturer's instructions. The PCR amplification conditions were as follows: Pre-denaturation at 95°C for 30 sec, followed by 40 cycles of amplification by denaturing at 95°C for 5 sec, annealing at 60°C for 30 sec and extending at 72°C for 30 sec. PCR cycling was performed using a Mx3005P qPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). The relative quantity of mRNA for each gene was normalized against the quantity of the housekeeping gene β-actin. Each sample was run and analyzed in triplicate. The primer sequences for CSE were as follows: 5'-GAG CCG GAG CAA TGG AGT TC-3' (forward) and 5'-GGA TTT CCA GAG CGG CTG TA-3' (reverse). The primer sequences for β-actin were: 5'-GGA GAT TAC TGC CCT GGC TCC TA-3' (forward) and 5'-GAC TCA TCG TAC TCC TGC TTG CTG-3' (reverse). The primers were designed and synthesized by Takara Biotechnology Co., Ltd.

Immunohistochemical analysis

Kidney tissues were fixed in 10% neutral formalin solution and then embedded in paraffin wax and cut into sections. Some sections were routinely stained with HE while other sections underwent deparaffinization, rehydration and inactivation, and were incubated with rabbit-anti-mouse CSE monoclonal antibody (1:50) at room temperature for 60 min, followed by incubation with secondary antibody (MaxVision™2) at room temperature for 15 min. The sections were mounted after staining. The primary anti-

body was replaced by phosphate-buffered saline to serve as a negative control. Five high-power microscopic fields were randomly chosen per slide and yellow material in the cytoplasm was considered to represent a CSE-positive cell. Cell staining was quantified as follows: > 75% positive cells scored 4; 50-75% positive cells scored 3; 25-50% positive cells scored 2; < 25% positive cells scored 1. Cell stain-

ing intensity was scored based on color as follows: no staining, 0; Faint yellow, 1; Light brown, 2; Dark brown, 3; The final score was defined as staining intensity × percentage of positive cells. The mean score of five fields was used to compare the three groups.

Statistical analysis

Data analysis was performed using SPSS 10.0 software (Chicago, IL, USA). Analysis of variance or Wilcoxon statistical methods were used to determine statistical significance. All measurements in this study are expressed as means ± SD. *P* < 0.05 was considered statistically significant.

Results

Biochemical examination

The serum levels of AST and ALT in the cirrhotic group were significantly higher than those in the sham group (*P* < 0.05, respectively). In addition, serum levels of Cre in the cirrhotic group were significantly higher than those in the sham group (*P* < 0.05), and the Ccr in the cirrhotic group was significantly lower than that in the sham group (*P* < 0.05). Compared with the cirrhotic group, the serum Cre levels were significantly lower (*P* < 0.05) and Ccr was higher in the NaSH-treated group (*P* > 0.05). The serum and urine concentrations of sodium in each experimental group were also measured. The serum and urine sodium concentrations were significantly lower in the cirrhotic group than in the sham group (*P* < 0.05, respectively).

Table 2. Effects of bile duct ligation and treatment with NaHS on hemodynamic parameters and 24-hour urine volume

	Sham group	Cirrhosis group	NaSH-treated group
PVP (mmHg)	9.26 ± 0.75	15.64 ± 2.38 ^a	13.64 ± 1.35
MAP (cmH ₂ O)	115.35 ± 8.21	58.37 ± 12.34 ^a	69.58 ± 7.78
RABF (mL/min·100 g)	3.93 ± 0.09	3.56 ± 0.04 ^a	3.71 ± 0.07 ^b
24-hour urine volume (ml/24 h)	15.10 ± 2.24	10.80 ± 1.87 ^a	12.30 ± 1.10 ^b

Values are expressed by mean ± SD (n = 8-10 for each group). PVP: portal vein pressure; MAP: mean arterial pressure; RABF: renal arterial blood flow. ^aP < 0.05 vs Sham group; ^bP < 0.05 vs Cirrhosis group.

Compared with the cirrhotic group, the serum and urine sodium concentration was significantly higher in the NaSH-treated group (P < 0.05, respectively). The levels of H₂S in arterial blood were significantly lower in the cirrhotic group than in the sham group (P < 0.05) and were significantly higher in the NaSH-treated group than in the cirrhotic group (P < 0.05) (Table 1).

Hemodynamic parameters and arterial blood gas levels

Compared with the sham group, the PVP was significantly higher and the MAP was significantly lower in the cirrhotic group (both P < 0.05). However, the PVP and the MAP were not significantly affected by treatment with NaSH compared with the cirrhotic group (P > 0.05). In addition, renal artery blood flow was significantly lower in the cirrhotic group than in the sham group (P < 0.05). Compared with the cirrhotic group, renal artery blood flow was significantly higher in the NaSH-treated group (P < 0.05). Furthermore, the 24-hour urine volume was significantly smaller in the cirrhotic group than in the sham group (P < 0.05). Compared with the cirrhotic group, the 24-hour urine volume was significantly larger in the NaSH-treated group (P < 0.05) (Table 2).

Histopathological analysis of the liver and kidney in cirrhotic and sham rats

Liver and kidney tissue samples from all three groups were stained with HE to examine the histopathological changes. In the livers of 4 weeks rats after BDL, we observed nodular regeneration of hepatocytes, collapse and disorganization of the hepatic lobular structure, lymphocytes infiltrating the portal area, and the formation of pseudolobuli surrounded by

fibrous septa. In contrast, except for congestion of the mesenchyme, there were no obvious pathological changes in the kidneys of the cirrhotic group as compared with those in the sham group (Figure 1).

Renal CSE mRNA expression levels

As determined by real time quantitative PCR, the renal expression level of CSE mRNA in the cirrhotic group was significantly lower than that in the sham group (P < 0.05). Compared with the cirrhotic group, renal CSE mRNA expression was significantly increased in the NaSH-treated group (P < 0.05) (Figure 2).

Immunohistochemical detection of CSE protein in the kidney

To localize the CSE protein expression in kidneys, we performed immunohistochemistry using specimens from all three groups. As shown in Figure 3, the expression of the CSE protein was mainly located in the proximal and distal convoluted tubule, part was located in the collecting pipe and its surrounding blood vessel, similar to that reported elsewhere [17, 18]. We also determined the intensity and percentage of cells expressing renal CSE protein. The CSE score in the cirrhotic group was significantly lower than that in the sham group (0.74 ± 0.25 vs 1.32 ± 0.36, P < 0.05). Compared with the cirrhotic group, the CSE score was significantly increased in the NaSH-treated group (1.28 ± 0.33 vs 0.74 ± 0.25, P < 0.05) (Figure 3).

Discussion

Our modified bile duct ligation (BDL) method has been proved as a reliable technique to establish biliary cirrhosis model. The change is that the bile duct was double ligated with 3-0 silk but not be completely scissored, which obviously decreased mortality. The study showed that the serum levels of AST and ALT were significantly higher in the cirrhotic group compared with the sham group, indicating that BDL had caused marked liver injury, and liver cirrhosis was confirmed by HE staining of the

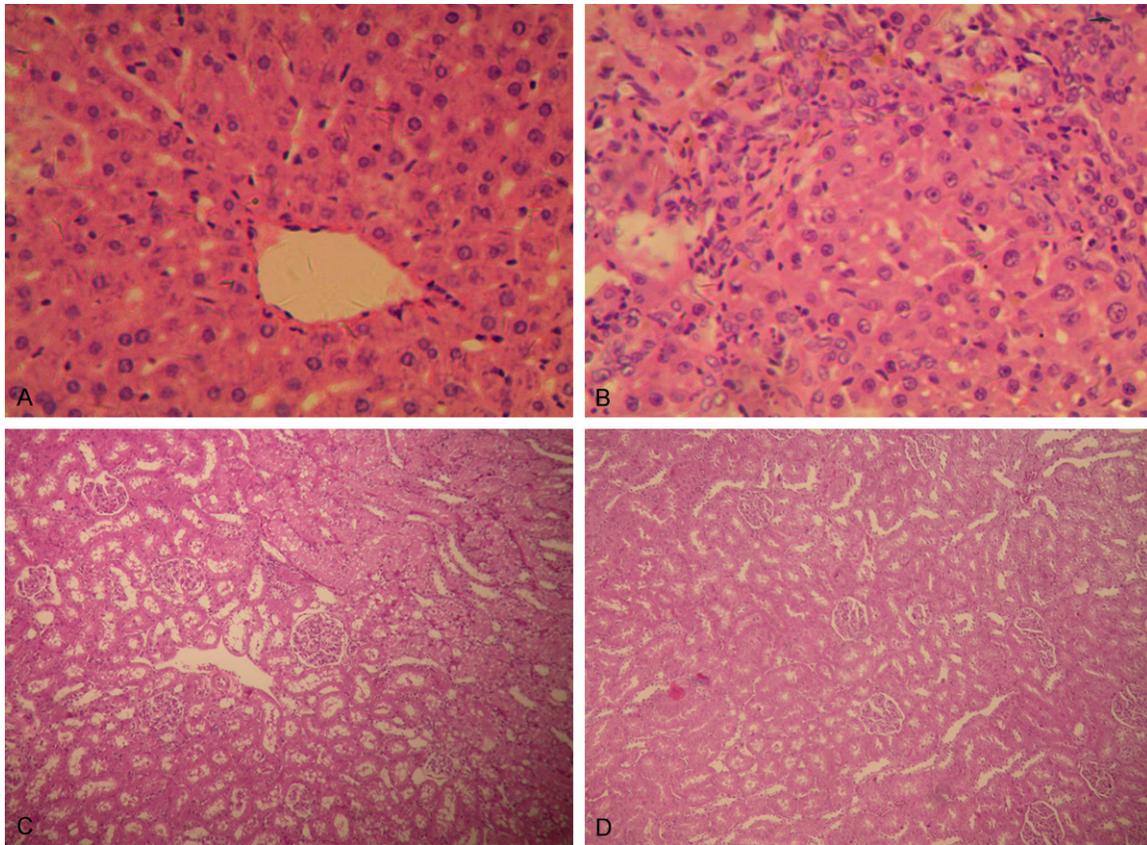


Figure 1. Representative photomicrographs showing pathological changes in the liver and kidney. A. Normal structure of liver; B. Liver cirrhosis; C. Normal structure of kidney; D. Renal structure in the cirrhotic group (magnification 200 ×; HE staining).

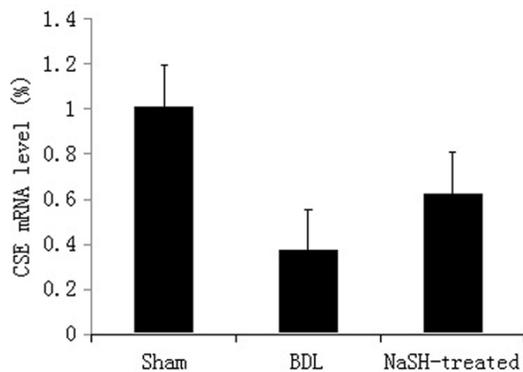


Figure 2. Renal CSE mRNA expression. Quantitative data showing the renal CSE mRNA expression levels. CSE mRNA levels were lower in the cirrhotic group than those in the sham group. Compared with the cirrhotic group, expression was significantly higher in the NaSH-treated group. ^aP < 0.05 vs Sham group; ^bP < 0.05 vs Cirrhosis group. CSE: cystathionine-γ-lyase.

liver specimens. In addition, compared with the sham group, MAP was significantly lower in the BDL group, which indicated the development of

a hyperdynamic state, and PVP was significantly increased in the BDL group, indicating the existence of portal hypertension. We also found renal dysfunction reflected by oliguria, hyponatremia, azotemia, and hyponatruiria in the cirrhotic group but there was no structure change in kidneys of cirrhotic rats.

Consistent with the renal dysfunction, the levels of H₂S in renal artery and renal CSE mRNA and protein levels were significantly lower in the cirrhotic group than in the sham group. H₂S has been acknowledged as the third endogenously produced gaseous signaling molecular after NO and CO, and has been shown to play important roles in regulating many physiological and pathophysiological processes, including vasodilation [12, 19, 20]. H₂S is generated from cysteine by pyridoxal-5'-phosphate-dependent enzymes, including cystathionine-β-synthase and cystathionine-γ-lyase (CSE), and CSE is the major contributor in kidneys [14, 21]. H₂S has cytoprotective effect, which could be attributed to its ability of neutralizing reactive oxygen spe-

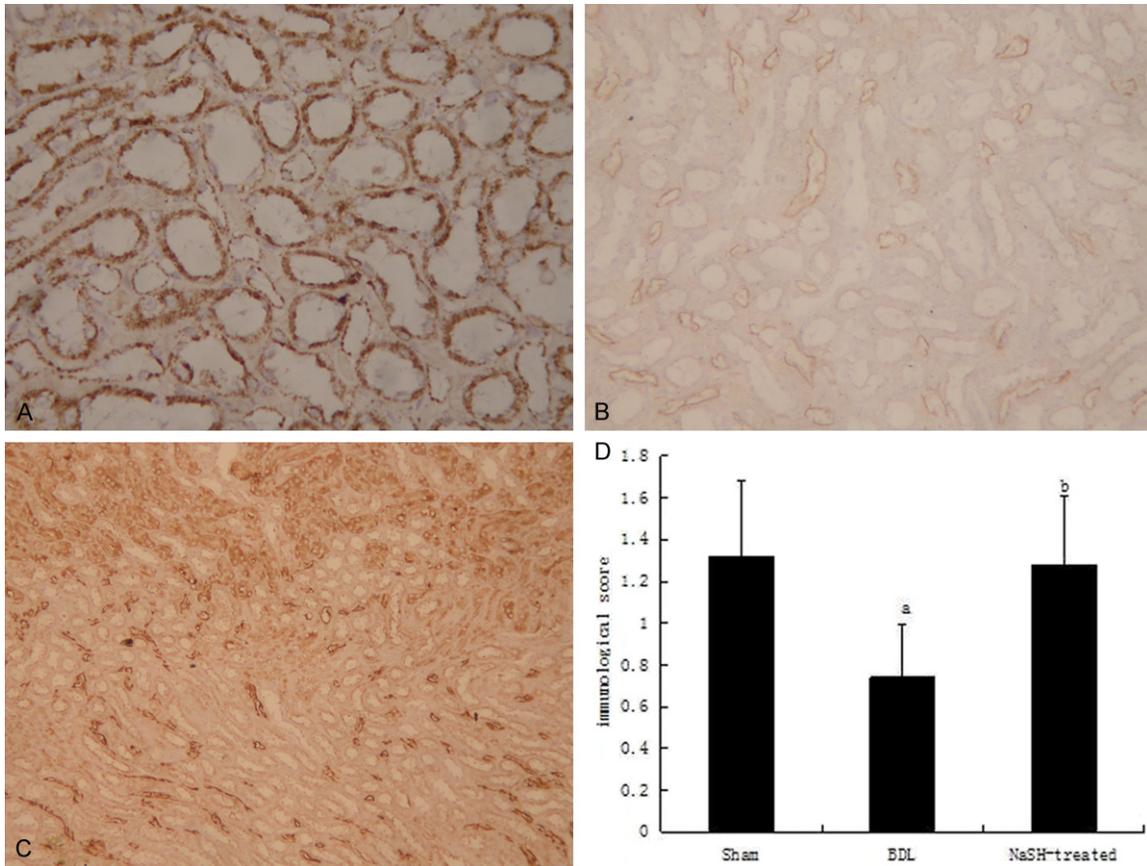


Figure 3. Renal CSE protein expression. (A-C) Immunohistochemical staining of renal CSE protein in rats in the sham group (A), the cirrhotic group (B), and the NaSH-treated group (C). And quantitative scoring (D) of immunohistochemical staining of renal CSE protein expression in each group. ^aP < 0.05 vs Sham group; ^bP < 0.05 vs Cirrhosis group. CSE: cystathionine- γ -lyase.

cies (ROS), reducing apoptotic signaling, reversibly modulating mitochondrial respiration, and promoting vascular smooth muscle relaxation [22]. H_2S relaxes blood vessel by activation of ATP-sensitive potassium channels and large-conductance Ca^{2+} -activated potassium channels in the vascular smooth muscle cells [12, 20], a different way from NO and CO [7, 23]. Many studies showed that exogenous and endogenous H_2S was protective against ischemia-reperfusion injury. During ischemia, H_2S level decreased along with renal function [24], administration of NaSH can improve renal function [25, 26], while inhibition of H_2S production worsen renal ischemia-reperfusion injury [27, 28]. However, the relationship between H_2S and renal function of cirrhotic rats is still not been elucidated. Since kidney is one of the major organs regulating endogenous H_2S production, in this study, we aimed to clarify the change of H_2S /CSE system in kidney and the effects of H_2S on renal function in cirrhotic rats.

Our study showed that accompanied with the decreased production of H_2S in renal artery and expression of renal CSE mRNA and protein, renal arterial blood flow and renal function were significantly decreased in the cirrhotic group than in the sham group. To further evaluate the functional consequences of H_2S generation changes, we treated BDL rats with NaHS, exogenous donor of H_2S . Compared with the cirrhotic group, NaHS treatment resulted in a significant increase in renal arterial blood flow and relieved renal dysfunction, as demonstrated by the restoration of 24-hour total urinary volume, serum and urine levels of sodium and Ccr, without obvious changes in PVP and MAP.

H_2S shares many characteristics with CO and NO, has been recognized as an important vascular dilation factor [12]. The kidney is a large supplier to total endogenous H_2S production, and H_2S appears to play an important physiological role in regulating blood flow and filtration

capacity of the kidney. The possible mechanism of protective effect of H₂S on renal function is induction of vasodilation [7]. In normal renal physiology, concentration of renal H₂S is correlating with increased renal blood flow (RBF) [4], and renal H₂S can increase urinary excretion, urinary sodium and urinary potassium by raising glomerular filtration rate (GFR) and inhibiting tubular sodium re-absorption [29]. Renal dysfunction in cirrhotic rats was attributed to renal vasoconstriction caused by reducing production of the endogenous vasodilator [4]. In our study, accompanied with the decreased production of renal H₂S, renal artery blood flow was significantly decreased in cirrhotic group compared with the sham group, while given exogenous H₂S by administration of NaHS significantly increased renal arterial blood flow and relieved renal dysfunction. In addition, oxidants also can cause localized renal vasoconstriction [30] and oxidative stress can promote the formation of a variety of vasoactive mediators, which decrease the glomerular capillary ultra-filtration coefficient [30]. As a major component of the endogenous anti-oxidant system [31, 32], H₂S can also decrease ROS production and simultaneously increase the antioxidant activity of glutathione [33, 34]. Furthermore, H₂S donors can reduce lipid peroxides, increase synthesis of glutathione [35, 36], and potentiate the antioxidant effects of glutathione [33]. So deficiency of H₂S contributes to the decreased renal arterial blood flow and renal dysfunction in cirrhotic rats.

In conclusion, our study showed that accompany with the decreased renal CSE expression and H₂S production, there was a significant reduction in renal arterial blood flow, Ccr, 24-hour total urinary volume, and serum and urine sodium concentrations in the cirrhotic group compared with those in the sham group. Administration of NaHS, exogenous donor of H₂S, can increase renal arterial blood flow and restore the renal dysfunction caused by the experimental model of HRS. Taken together, these results indicate that HRS is associated with and, in part, mediated by decreased H₂S production and CSE expression in the kidney in experimental animals, and exogenous H₂S can ameliorate renal function in cirrhotic rats.

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Disclosure of conflict of interest

None.

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