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Original Article

Hydrogen Sulfide Promotes Platelet Autophagy via PDGFR-α/PI3K/Akt Signaling in Cirrhotic Thrombocytopenia

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Abstract

Background and Aims: The role of platelet autophagy in cirrhotic thrombocytopenia (CTP) remains unclear. This study aimed to investigate the impact of platelet autophagy in CTP and elucidate the regulatory mechanism of hydrogen sulfide (H_2S) on platelet autophagy. ***Methods:*** Platelets from 56 cirrhotic patients and 56 healthy individuals were isolated for *in vitro* analyses. Autophagy markers (ATG7, BECN1, LC3, and SQSTM1) were quantified using enzyme-linked immunosorbent assay, while autophagosomes were visualized through electron microscopy. Western blotting was used to assess the autophagy-related proteins and the PDGFR/PI3K/Akt/mTOR pathway following treatment with NaHS (an H_2S donor), hydroxocobalamin (an H_2S scavenger), or AG 1295 (a selective PDGFR-α inhibitor). A carbon tetrachloride-induced cirrhotic BALB/c mouse model was established. Cirrhotic mice with thrombocytopenia were randomly treated with normal saline, NaHS, or hydroxocobalamin for 15 days. Changes in platelet count and aggregation rate were observed every three days. ***Results:*** Cirrhotic patients with thrombocytopenia exhibited significantly decreased platelet autophagy markers and endogenous H_2S levels, alongside increased platelet aggregation, compared to healthy controls. *In vitro,* NaHS treatment of platelets from severe CTP patients elevated LC3-II levels, reduced SQSTM1 levels, and decreased platelet aggregation in a dose-dependent manner. H_2S treatment inhibited PDGFR, PI3K, Akt, and mTOR phosphorylation. *In vivo*, NaHS significantly increased LC3-II and decreased SQSTM1 expressions in platelets of cirrhotic mice, reducing platelet aggregation without affecting the platelet count. ***Conclusions:*** Diminished platelet autophagy potentially contributes to thrombocytopenia in cirrhotic patients. H_2S modulates platelet autophagy and functions possibly via the PDGFR-α/PI3K/Akt/mTOR signaling pathway.

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Keywords: Liver cirrhosis; Thrombocytopenia; Platelet; Autophagy; Hydrogen sulfide; NaHS; PDGFR-α; PI3K/Akt/mTOR signaling.

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Introduction

Chronic liver disease-related thrombocytopenia (CLD-TP) is defined as a decrease in platelet count ($<150\times10^9$/L internationally or $<100\times10^9$/L in China) caused by the liver disease itself or liver disease-related treatments in patients with chronic liver diseases.[1–3] The incidence of CLD-TP in chronic hepatitis ranges from 6% to 16%, and it can even reach 78% in patients with liver cirrhosis.[2] CLD-TP is caused by a complex pathophysiological process involving multiple mechanisms, including decreased platelet production, increased disruption, and abnormal distribution.[3,4] Specifically, cirrhotic thrombocytopenia (CTP), a common complication of liver cirrhosis, accounts for nearly 80% of cases in cirrhotic patients and is significantly associated with bleeding events in these patients.[3,4] However, the exact molecular mechanisms underlying the pathophysiology of CTP are largely unknown.

Autophagy is the process of transporting intracellular "waste", such as aging or damaged organelles and misfolded proteins, to lysosomes for degradation to maintain cellular homeostasis and energy metabolism.[5,6] Previous research on autophagy predominantly focused on nucleated cells, but recent studies have confirmed the autophagy mechanism in anucleated platelets. Feng *et al*. were the first to systematically elucidate the constitutive expression of the autophagy mechanism in human platelets, demonstrating its necessity for maintaining their fundamental functions.[7] Moreover, it was shown that inhibiting autophagic degradation with the lysosome inhibitor Baf-A1 impacts platelet aggregation and adhesion.[7,8] However, evidence of platelet autophagy's role in the clinical context of CTP remains elusive.

Hydrogen sulfide (H_2S) is a recently discovered endogenous signaling molecule that regulates various pathophysiological mechanisms such as inflammation and angiogenesis.[9,10] It was reported that the concentration of H_2S in the portal vein blood of cirrhotic rats was significantly decreased.[11] Increasing evidence demonstrated that H_2S could regulate autophagy, cellular apoptosis, and metabolism.[12,13] However, the regulatory role of H_2S in platelet autophagy is still unclear. This study aimed to investigate the impact of platelet autophagy in CTP and delineate the regulatory mechanism of H_2S on platelet autophagy.

Mthods

Study population

This study included 56 cirrhotic patients, with or without

thrombocytopenia, who visited Beijing You'an Hospital between September 2022 and February 2023. Additionally, 56 healthy blood donors who volunteered during the same period were included in the control group. All participants provided written informed consent. The Ethics Committee of Beijing You'an Hospital approved this study, which was conducted in accordance with the Declaration of Helsinki guidelines. The Strengthening the Reporting of Observational Studies in Epidemiology guidelines were applied to the observational clinical component of this study.

Human platelet extraction and preparation

Whole blood was collected from all subjects (n=112) in blood collection tubes containing sodium citrate. Then, 5 mL of platelet isolation solution (Solarbio, P6390) was added, followed by centrifugation at 200 g for 15 m to collect platelet-rich plasma (PRP). Subsequently, 10 mL of platelet detergent (Solarbio, P6390) was added to the PRP, and the mixture was centrifuged at 1,000 g for 20 m to obtain platelet pellets. After washing, platelet pellets were suspended in modified Tyrode's buffer (Solarbio, T1420) for subsequent experiments. Platelet counts were measured using a HemaVet cell counter (Drew Scientific, USA).

Enzyme-linked immunosorbent assay (ELISA) for in vitro experiments

Platelets from healthy controls and liver cirrhosis patients were isolated using the aforementioned method, followed by the addition of cell lysis buffer for adequate lysis. The platelet lysate was centrifuged at 12,000 g for 5 m. The supernatant was collected for ELISA assays to measure the levels of ATG7 (Human ATG7 ELISA Kit, Proteintech KE00276), BECN1 (Human Beclin 1 ELISA Kit, ABclonal RK00973), LC3 (Total LC3B ELISA Kit, Cell Signaling Technology #35172), and SQSTM1 (Human SQSTM1/Sequestosome-1 ELISA Kit, ABclonal RK04613). Additionally, serum isolated from healthy controls and cirrhotic patients was analyzed to determine PDGF-BB levels using ELISA kits (Human PDGF-BB ELISA Kit, Proteintech KE00161). All assays were conducted according to the manufacturer's guidelines. Absorbance readings at 450 nm were performed using a microplate reader (Bio-Rad Laboratories Inc., CA, USA). Protein concentrations were determined by comparing their absorbance to the standards and plotting a standard curve.

Detection of endogenous H_2S in serum

The serum from healthy controls and patients with cirrhosis was isolated using the above method. Then, 100 μL of serum was taken, and 1 mL of H_2S extraction reagent (Solarbio, BC2055) was added to it. The mixture was then centrifuged at 12,000 g for 10 m. According to the instructions of the H_2S content detection kit (Solarbio, BC2055), the samples and H_2S detection reagents were added to 96-well plates. The absorbance values of each well were measured at 665 nm on a microplate reader (Bio-Rad Laboratories Inc., CA, USA). The H_2S concentration in the samples was calculated according to the standard curve.

Transmission electron microscopy

Human platelets were affixed to microscopy slides and fixed with 2.5% glutaraldehyde (Sigma Aldrich, 104239) and 1% osmium tetroxide (Sigma Aldrich, 75633) for 2 h. The samples underwent sequential dehydration using 70%, 90%, and 100% acetone solutions (Supelco, 48358). EMBed 812 (SPI, 90529-77-4) was used for complete embedding, and the embedding plate was baked at 60°C for 24 h. Resin blocks were sectioned into 50 nm thin slices with an ultramicrotome (Leica EM UC7), and samples were placed on 150 mesh copper grids coated with formvar film (Servicebio, WFHM-150). Samples were stained with a 2% uranium acetate-saturated alcohol solution (Wengjiang, 541-09-3) for 8 m, avoiding light exposure, followed by rinsing three times in 70% ethanol and three times in ultra-pure water. Afterward, 2.6% lead citrate (Headbio, HD17800) was applied for 8 m to prevent CO_2 staining, followed by three rinses with ultra-pure water. The copper grids were then examined under an electron microscope (Hitachi, HT7700).

Detection of autophagy markers after treatment with NaHS

Platelets were isolated from patients with severe thrombocytopenia using the aforementioned method and treated with NaHS (H_2S donor) at different concentration gradients (0 μM, 25 μM, 50 μM, 100 μM) for 4 h. The expression levels of autophagy markers in platelets treated with NaHS were analyzed by Western blot.

Detection of platelet reactive oxygen species (ROS) in vitro

The platelet ROS was detected with the Reactive Oxygen Species Assay Kit (Solarbio, CA1410). Platelets were isolated and resuspended with diluted DCFH-DA (10 μmol/L) followed by incubation at 37°C for 30 m. Platelets were rinsed with Tyrode's buffer (Solarbio, T1420) and treated with the H_2S donor (NaHS) (Sigma-Aldrich, 161527-5G) or the H_2S scavenger hydroxocobalamin (HY-B2209) for 4 h. A microplate reader was used to measure the absorbance value at 488 nm (Bio-Rad Laboratories Inc., CA, USA).

CellTiter-Glo (CTG) assay of platelet viability in vitro

We used the CTG Luminescent Cell Viability Assay Kit (Promega, G7572) to detect the cell viability of platelets. Platelets were diluted with Tyrode's buffer (Solarbio, T1420) and treated with the H_2S donor NaHS (Sigma-Aldrich, 161527-5G) or the H_2S scavenger hydroxocobalamin (HY-B2209) for 4 h. Add 100 μL of sample and 100 μL of CellTiter Glo Reagent to each well in 96-well plates and mix for 2 m. After incubating at room temperature for 10 m, a microplate reader was employed to measure the absorbance value at 450 nm (Bio-Rad Laboratories Inc., CA, USA).

Platelet apoptosis detection in vitro

The platelet apoptosis was detected using the Annexin V Alexa Fluor488/PI apoptosis detection kit (Solarbio, CA1040). Platelets were diluted with Tyrode's buffer (Solarbio, T1420) and treated with the H_2S donor NaHS (Sigma-Aldrich, 161527-5G) or the H_2S scavenger hydroxocobalamin (HY-B2209) for 4 h. Approximately 100 μL of platelet suspension was taken in a flow cytometry tube, and 5 μL of Annexin V was added, followed by incubation in the dark for 5 m. Then, 5 μL of PI and 400 μL of PBS were added. Fluorescence was measured within 30 m on a FACS Calibur (Becton Dickinson). The data were analyzed by FlowJo Software.

Establishment of a CCl_4-induced cirrhotic mouse model

BALB/c mice were purchased and maintained at Geneline Bioscience (Beijing, China). All procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (8th edition, 2011). This study followed the ARRIVE

guidelines for the preclinical experimental part of this study. Forty mice were intraperitoneally injected with a 20% carbon tetrachloride solution of 4 mL/kg twice a week for 14 weeks.[14] After 14 weeks of injection, four mice were randomly euthanized, and their liver tissues were taken for pathological biopsy to confirm the successful establishment of the cirrhotic mouse model. Cirrhotic mice with platelet counts ranging from 100–150×10^9/ml (normal value 150–500×10^9/ml) were selected and randomly divided into five groups. Each group was continuously injected with normal saline, H_2S donor (NaHS) (Sigma-Aldrich, 161527-5G), or H_2S scavenger hydroxocobalamin (HY-B2209) for 15 days, respectively. The changes in platelet count and platelet aggregation rate were observed every three days.

Mouse platelet extraction and preparation

Mouse blood (0.5 mL) was collected via cardiac puncture into centrifuge tubes containing sodium citrate. PRP was obtained after centrifugation at 200 g for 15 m. To the PRP, 3 mL of platelet lysis buffer (Solarbio, P6390) was added, followed by centrifugation at 1,000 g for 20 m to yield platelet pellets. These pellets were then washed and resuspended in modified Tyrode's buffer (Solarbio, T1420) for subsequent experiments.

Western blotting for both in vitro and in vivo experiments

Washed platelets (1.0×10^9/mL) were incubated with NaHS (Sigma-Aldrich, 161527-100G), PDGF-BB (MedChemExpress, HY-P7055), hydroxycobalamin (MedChemExpress, HY-B2209), AG 1295 (MedChemExpress, HY-101957), respectively, or in combination. A platelet protein extraction kit (Solarbio, EX1210) was used to lyse platelets, and electrophoresis of the lysed platelet protein was conducted on SDS polyacrylamide gel. The protein was then transferred onto PVDF membranes (Millipore, IPVH00010). After blocking with 5% BSA (Solarbio, SW3015) in TBST (150 mM NaCl, 10 mM TRIS-HCl, and 0.1% Tween 20), the PVDF membranes were incubated with the following antibodies: LC3 Antibody (Cell Signaling Technology, #2775), SQSTM1 Antibody (Cell Signaling Technology, #5114), PI3 Kinase Class III Antibody (Cell Signaling Technology, #3811); Phospho-PI3 Kinase Class III (Ser249) Antibody (Cell Signaling Technology, #13857), AKT monoclonal antibody (Proteintech, 60203-2-Ig), Phospho-AKT (Ser473) polyclonal antibody (Proteintech, 28731-1-AP), mTOR monoclonal antibody (Proteintech, 66888-1-Ig), Phospho-mTOR (Ser2448) monoclonal antibody (Proteintech, 67778-1-Ig), PDGF Receptor α monoclonal antibody (Cell Signaling Technology, #3164), Phospho-PDGF Receptor α (Tyr762) monoclonal antibody (Cell Signaling Technology, #24188), β-actin monoclonal antibody (Proteintech, 66009-1-Ig). After rinsing the PVDF membranes with TBST, the membranes were incubated with HRP-conjugated Affinipure Goat Anti-Rabbit IgG (Proteintech, SA00001-2) or HRP-conjugated Affinipure Goat Anti-Mouse IgG (Proteintech, SA00001-1). Immunoblots were visualized with ECL chemiluminescence detection reagents (Solarbio, SW2050). The signal intensity of immunoblotting was quantified using Image J.

Detection of platelet aggregation rate in vitro and in vivo

As described previously, the AggRAM Platelet Aggregometer (Helena, USA) was used to measure platelet aggregation.[15] Platelet suspensions (3.0×10^8/mL) were stimulated using ADP (2 μmol/L) or arachidonic acid (1 mmol/L) for 10 m; the extent of aggregation was expressed in light transmission units.

Statistical analysis

All statistical data are presented as mean±standard error of the mean. The Student's t-test for continuous data and the chi-square test for categorical data were used to compare the parameters of the two groups. A *p*-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 22.0.

Results

Baseline characteristics of study participants

The clinical information for cirrhotic patients and healthy volunteers included in the study is detailed in Table 1.

Platelet autophagy and serum endogenous H_2S levels in patients with CTP

The ELISA assay revealed that platelet autophagy biomarkers in cirrhotic patients were significantly reduced compared to those in healthy controls (Fig. 1A). Additionally, the levels of autophagy biomarkers correlated negatively with the Child-Pugh score and the severity of thrombocytopenia (Fig. 1B, C). Transmission electron microscopy demonstrated that CTP patients had significantly fewer platelet autophagosomes compared to healthy controls, with levels of autophagosomes correlating negatively with the severity of thrombocytopenia (Fig. 1D). Serum endogenous H_2S levels in CTP patients were also significantly reduced compared to healthy controls, and these levels correlated negatively with the severity of thrombocytopenia (Fig. 1E). Spearman correlation analysis indicated that in CTP patients, serum H_2S levels positively correlated with platelet autophagy markers LC3 and ATG7 and negatively correlated with SQSTM1 (Fig. 1F).

H_2S promoted platelet autophagy

NaHS increased relative LC3-II expression and attenuated SQSTM1 expression dose-dependently (Fig. 2A). To further investigate the effect of H_2S on platelet autophagic flux, we treated platelets with Baf A1 (an autophagy inhibitor) or 100 μM NaHS alone or in combination. Western blot results showed that compared with Baf A1 treatment alone, LC3-II expression significantly increased after NaHS+Baf A1 treatment (Fig. 2B), indicating that H_2S increased platelet autophagosome synthesis. Compared with the negative control, after treating platelets with 100 μM NaHS, LC3-II expression increased and SQSTM1 expression decreased (Fig. 2B). These results suggest that H_2S could increase platelet autophagy in patients with severe thrombocytopenia.

H_2S down-regulated PDGFR/PI3K/Akt signaling pathway to promote platelet autophagy

NaHS inhibited the phosphorylation of PI3K, Akt, and mTOR in a dose-dependent manner without affecting total protein levels, thus promoting platelet autophagy (Fig. 3A). Serum PDGF-BB levels were measured in healthy controls and cirrhotic patients, revealing significantly higher levels in the latter (Supplementary Fig. 1). To assess the impact of PDGFR on the PI3K/Akt signaling pathway, platelets from patients with severe thrombocytopenia were treated with human PDGF-BB recombinant protein for 4 h. This treatment significantly increased the phosphorylation of PDGFR and the PI3K/Akt/mTOR proteins (Fig. 3B). Subsequently, to verify whether NaHS affected the PDGFR/PI3K signaling pathway,

Table 1. Clinical characteristics of patients and healthy volunteers

Characteristic		Patients (n=56)	Healthy volunteers (n=56)	*p*-value
Gender [N (%)]	Males	24 (42.8)	39 (69.6)	<0.05
	Females	32 (57.2)	17 (30.4)	
Age [P_{50} (P_{25}, P_{75})]		52 (42, 67)	34 (22, 51)	<0.05
Etiology of liver disease [N (%)]	Hepatitis B cirrhosis	56 (100)		
Child-Pugh [N (%)]	A	16 (28.6)		
	B	23 (41.0)		
	C	17 (30.4)		
Platelet count (×10^9/L)	Normal (>100)	12 (21.4)	214.24±62.53	
	Mild TP (75–100)	11 (19.6)		
	Moderate TP (50–75)	20 (35.7)		
	Severe TP (<50)	13 (23.3)		
RBC (×10^{12}/L)		3.54±1.41	4.42±1.64	<0.05
WBC (×10^9/L)		4.51±1.79	7.63±2.45	<0.05
APTT (s)		35.66±7.02	27.32±6.47	<0.05
PT (s)		15.32±3.25	12.41±2.89	<0.05
TT (s)		16.59±3.47	13.64±3.96	<0.05
FIB (g/L)		2.18±0.75	3.43±1.21	<0.05
ALB (g/L)		34.26±6.21	46.32±5.30	<0.05
ALT (U/L)		87.00 (62.00, 112.50)	25.50 (19.00, 36.00)	<0.05
AST (U/L)		32.50 (23.00, 49.00)	22.00 (17.00, 31.00)	<0.05
TBIL (μmol/L)		24.45 (18.15, 35.50)	11.65 (7.40, 16.35)	<0.05

TP, thrombocytopenia; RBC, red blood cell count; WBC, white blood cell count; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; FIB, fibrinogen; ALB, albumin; ALT, alanine aminotransferase; AST, Aspartate aminotransferase; TBIL, total bilirubin.

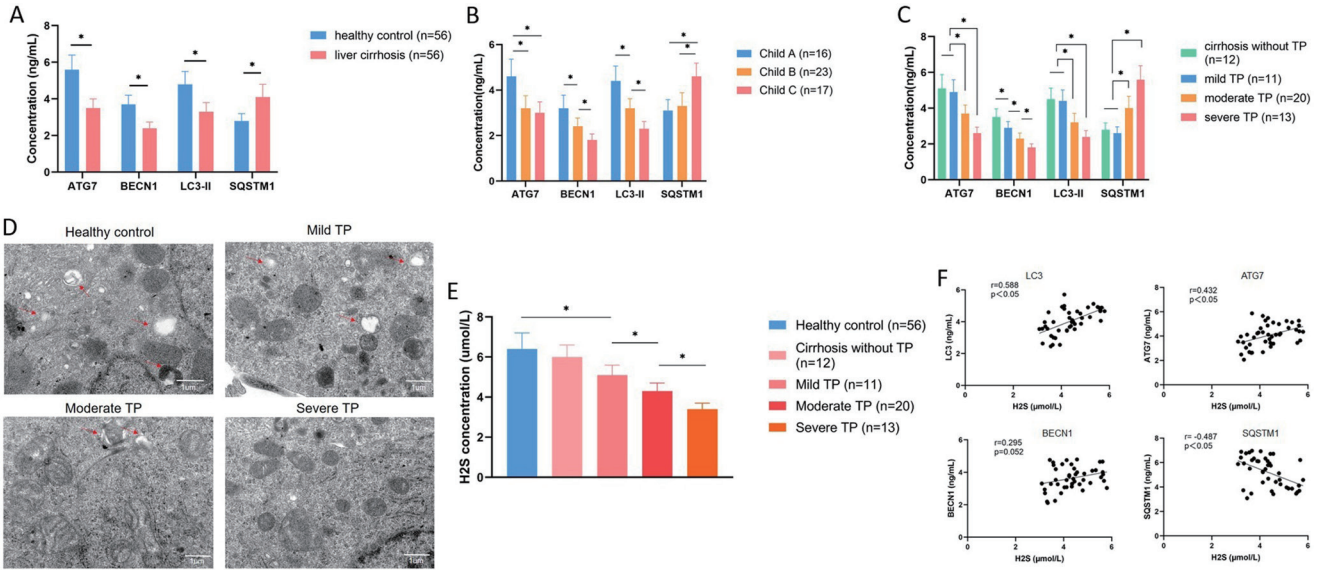

Fig. 1. Platelet autophagy and serum endogenous H_2S levels in cirrhotic patients with thrombocytopenia (TP). (A) Enzyme-linked immunosorbent assay (ELISA assay) showed that the platelet autophagy biomarkers in cirrhotic patients were significantly reduced compared to the healthy controls. (B–C) The levels of autophagy biomarkers negatively correlated with the Child-Pugh score and the severity of TP. (D) Transmission electron microscopy showed that TP patients had significantly reduced platelet autophagosomes compared to healthy controls. (E) The serum endogenous H_2S levels in TP patients were significantly reduced compared to healthy controls. (F) Spearman correlation analysis indicated that serum H_2S levels positively correlated with platelet autophagy markers LC3 and ATG7 and negatively correlated with SQSTM1 in TP patients. * indicates $p<0.05$. Scale bar: 1 μm.

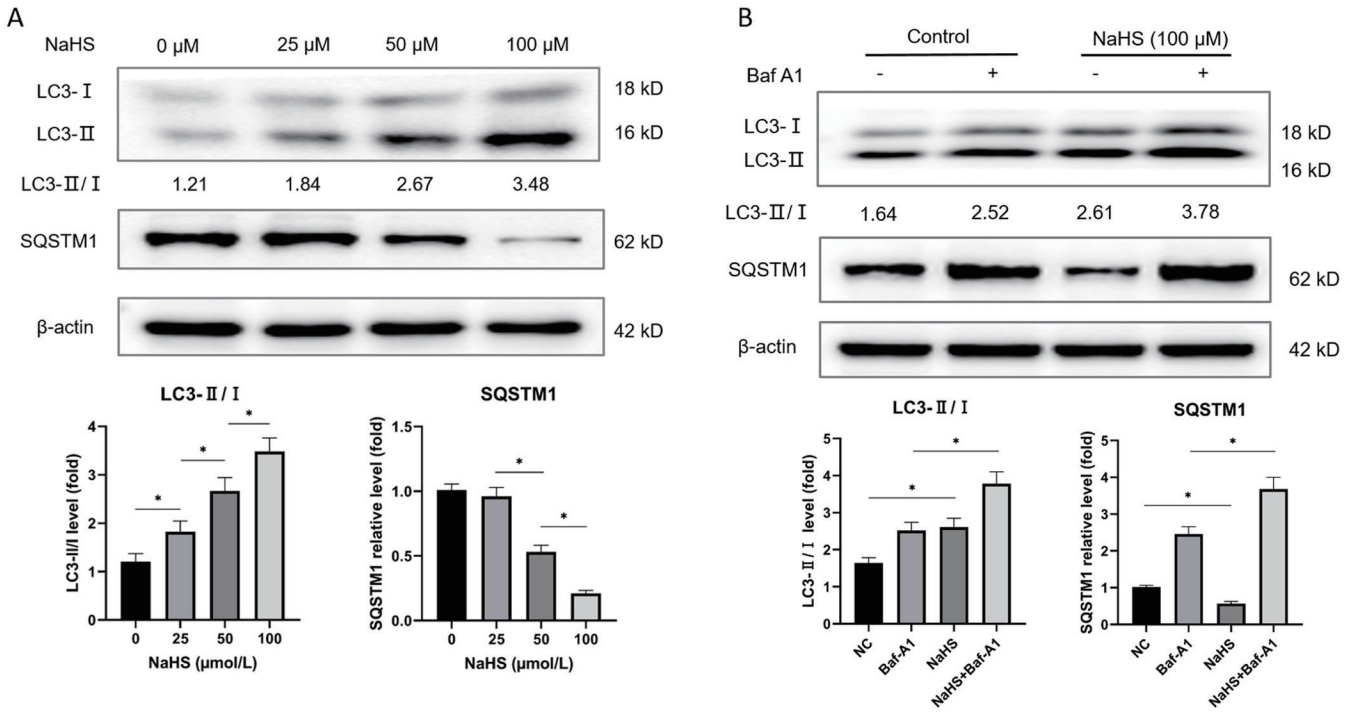

Fig. 2. H_2S promoted platelet autophagy. (A) NaHS increased the relative LC3-II expression and attenuated the SQSTM1 expression dose-dependently. (B) Compared with the Baf A1 treatment alone, the LC3-II expression was significantly increased after NaHS+Baf A1 treatment indicated that the platelet autophagosome synthesis was increased by H_2S. * indicates $p<0.05$. "+" indicates using Bafilomycin (Baf) A1 treatment, "−" indicates using culture medium treatment. NC, negative control.

we treated platelets with 100 μM NaHS with or without hydroxocobalamin (HC, an H_2S scavenger) and used AG 1295 (a selective PDGFR-α inhibitor) as a positive control. Western blot results showed that NaHS inhibited the phosphorylation of PDGFR/PI3K/Akt/mTOR proteins, and the addition of HC could reverse this effect (Fig. 3C). This evidence indicated that H_2S down-regulated the PDGFR/PI3K/Akt signaling pathway to promote platelet autophagy.

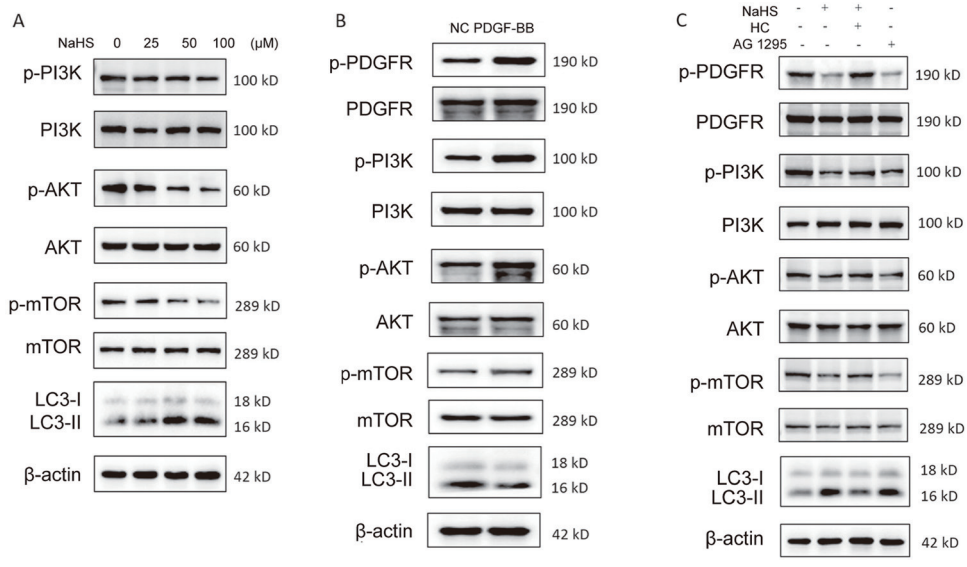

Fig. 3. H_2S down-regulated PDGFR/PI3K/Akt signaling pathway to promote platelet autophagy. (A) NaHS inhibited the phosphorylation of PI3K, Akt, and mTOR in a dose-dependent manner without affecting the total protein expression. (B) On treatment of platelets of severe thrombocytopenia patients with human PDGF-BB recombinant protein, the phosphorylation of PDGFR and PI3K/Akt/mTOR proteins were significantly augmented. (C) Platelets were treated with 100 μM NaHS with or without hydroxocobalamin (HC, an H_2S scavenger) and AG 1295 (a selective PDGFR-α inhibitor) as a positive control. Western blot results showed that NaHS inhibited the phosphorylation of PDGFR/PI3K/Akt/mTOR proteins. The addition of HC could reverse this effect. "+" indicates adding NaHS, HC or AG1295 treatment, "−" indicates adding culture medium treatment. NC, negative control; PDGF-BB, platelet derived growth factor-BB.

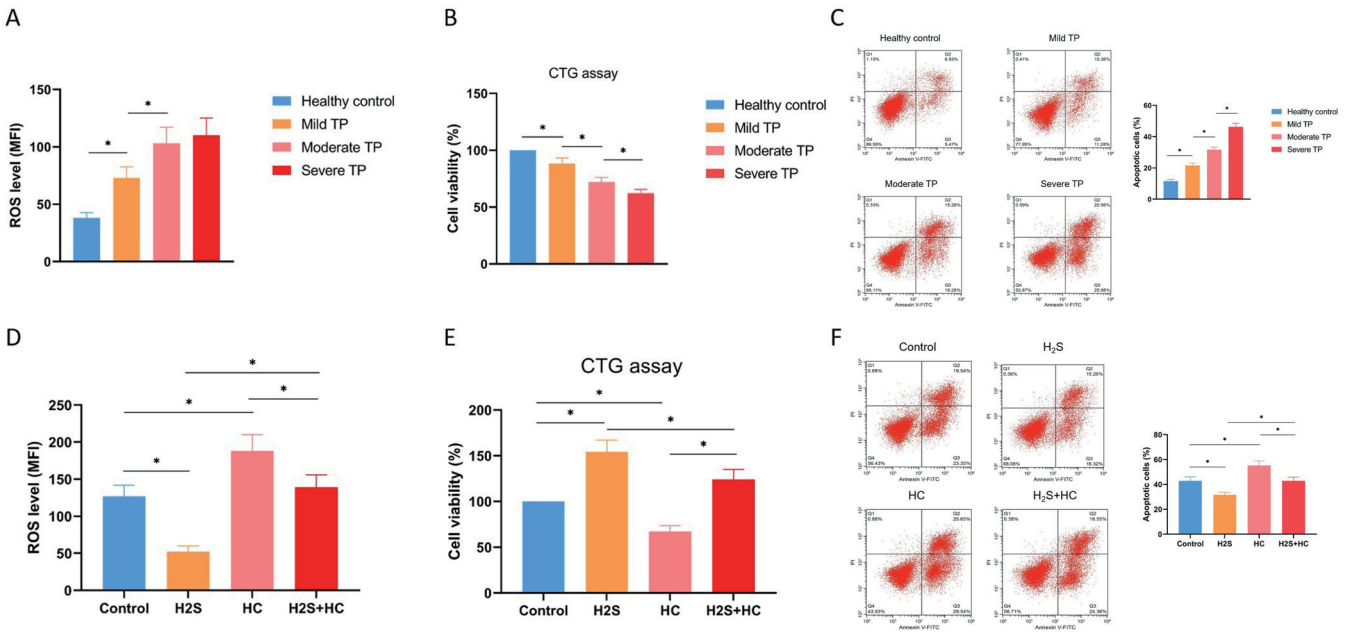

Fig. 4. H_2S affected intracellular reactive oxygen species (ROS), cell viability, and apoptosis in platelets by regulating autophagy. (A) The intracellular ROS level in platelets of thrombocytopenia (TP) was significantly higher than that of healthy controls, and it increased with increasing severity of thrombocytopenia. (B) The platelet viability of TP was significantly lower than that of healthy controls, and it decreased with increasing severity of TP. (C) The platelet apoptosis in TP was significantly higher than that in healthy controls, and it increased with increasing severity of TP. (D) After treatment with 100 μM NaHS (H_2S donor), the intracellular ROS level of platelets in severe TP patients was significantly reduced, while after treatment with hydroxocobalamin (HC, H_2S scavenger), it was significantly increased. (E) CellTiter-Glo (CTG) assay indicated that after treatment with NaHS, the platelet viability in severe TP patients was significantly increased, while on treatment with HC, their viability was significantly inhibited. (F) The flow cytometry showed that the proportion of apoptotic platelets in severe TP patients significantly decreased after NaHS treatment and increased after HC treatment. * indicates $p<0.05$.

H_2S affected intracellular ROS, cell viability, and apoptosis in platelets by regulating autophagy

The intracellular ROS level in platelets of patients with CTP was significantly higher than that of healthy controls, increasing with the severity of thrombocytopenia. However, platelet ROS levels in patients with moderate and severe thrombocytopenia were similar (Fig. 4A). The platelet viability in CTP was significantly lower than in healthy controls, decreasing with increasing severity of thrombocytopenia (Fig. 4B). Moreover, the platelet apoptosis in CTP was significantly higher than in healthy controls, increasing with increasing severity of thrombocytopenia (Fig. 4C). Treatment with 100 μM NaHS (H_2S donor) significantly reduced intracellular ROS levels in platelets of severe CTP patients, while treatment with HC (H_2S scavenger) significantly increased ROS levels (Fig. 4D). The CTG assay indicated that NaHS treatment significantly increased platelet viability in severe CTP patients, while HC treatment significantly inhibited viability (Fig. 4E). The flow cytometry showed that the proportion of apoptotic platelets in severe CTP patients significantly decreased after NaHS treatment and increased after HC treatment (Fig. 4F). This evidence indicated that H_2S reduced intracellular ROS levels, enhanced platelet viability, and inhibited platelet apoptosis by regulating platelet autophagy.

H_2S inhibited platelet aggregation in CTP patients in vitro

The platelet aggregation rate in CTP patients was significantly higher than in healthy controls and escalated with the severity of thrombocytopenia. Nonetheless, platelet aggregation rates in moderate and severe CTP patients were comparable (Fig. 5A). In mild CTP patients, only 100 μM NaHS significantly reduced the platelet aggregation rate. For moderate or severe CTP patients, NaHS decreased the platelet aggregation rate dose-dependently (Fig. 5B). In these patients, 100 μM NaHS treatment significantly reduced the platelet aggregation rate, which then increased following HC treatment (Fig. 5C).

H_2S promoted platelet autophagy and reduced platelet aggregation in vivo

A pathologically confirmed liver cirrhosis mouse model was developed as described previously, and mice with low platelet counts ($100–150\times10^9$/L) were selected and randomized into five groups. Changes in platelet count and aggregation rate were observed after injection of the indicated reagents for 15 days (Fig. 6A). Platelets were isolated from mice, and autophagy markers were determined by Western blot. NaHS injection increased the platelet LC3-II expression and reduced the SQSTM1 expression in a dose-dependent manner (Fig. 6B). After HC (H_2S scavenger) injection, platelet LC3-II expression significantly decreased, whereas SQSTM1 expression significantly increased (Fig. 6B). Injection with varying concentrations of NaHS or HC did not significantly alter the platelet count in mice at the indicated times (Fig. 6C). However, the platelet aggregation rate was significantly reduced by Day 15 (Fig. 6D). In summary, H_2S can promote platelet autophagy and decrease the platelet aggregation rate *in vivo*.

Discussion

Traditionally, thrombocytopenia in cirrhotic patients was believed to stem from decreased endogenous thrombopoietin

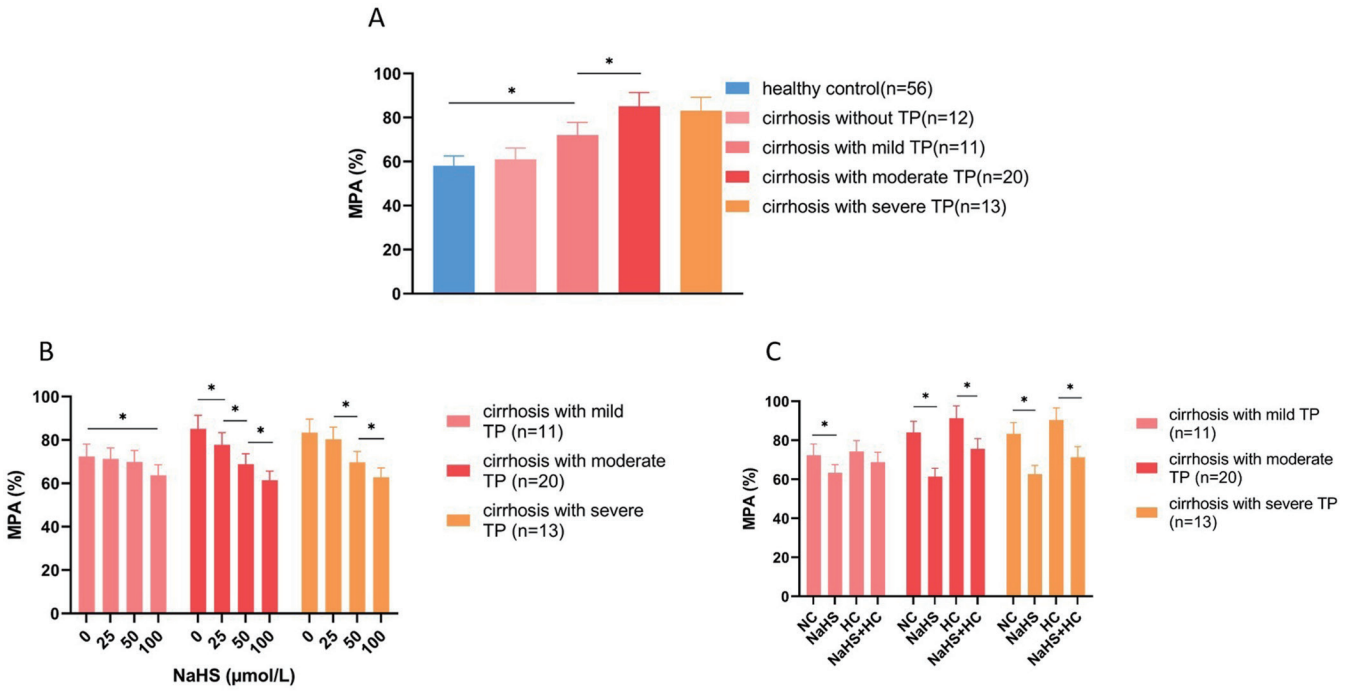

Fig. 5. H_2S inhibited platelet aggregation in cirrhotic patients with thrombocytopenia (TP) *in vitro*. (A) The platelet aggregation rate of patients with TP was significantly higher than that of healthy controls, and it increased with increasing severity of TP. However, the platelet aggregation rate of moderate and severe TP patients was similar. (B) In mild TP patients, the platelet aggregation rate was significantly reduced by 100 μM NaHS only. In patients with moderate or severe TP, NaHS significantly reduced platelet aggregation rate in a dose-dependent manner. (C) In moderate and severe TP patients, the platelet aggregation rate was significantly decreased after 100 μM NaHS treatment and increased after hydroxocobalamin treatment. * indicates $p<0.05$. NC, negative control; MPA, microscopic polyangiitis.

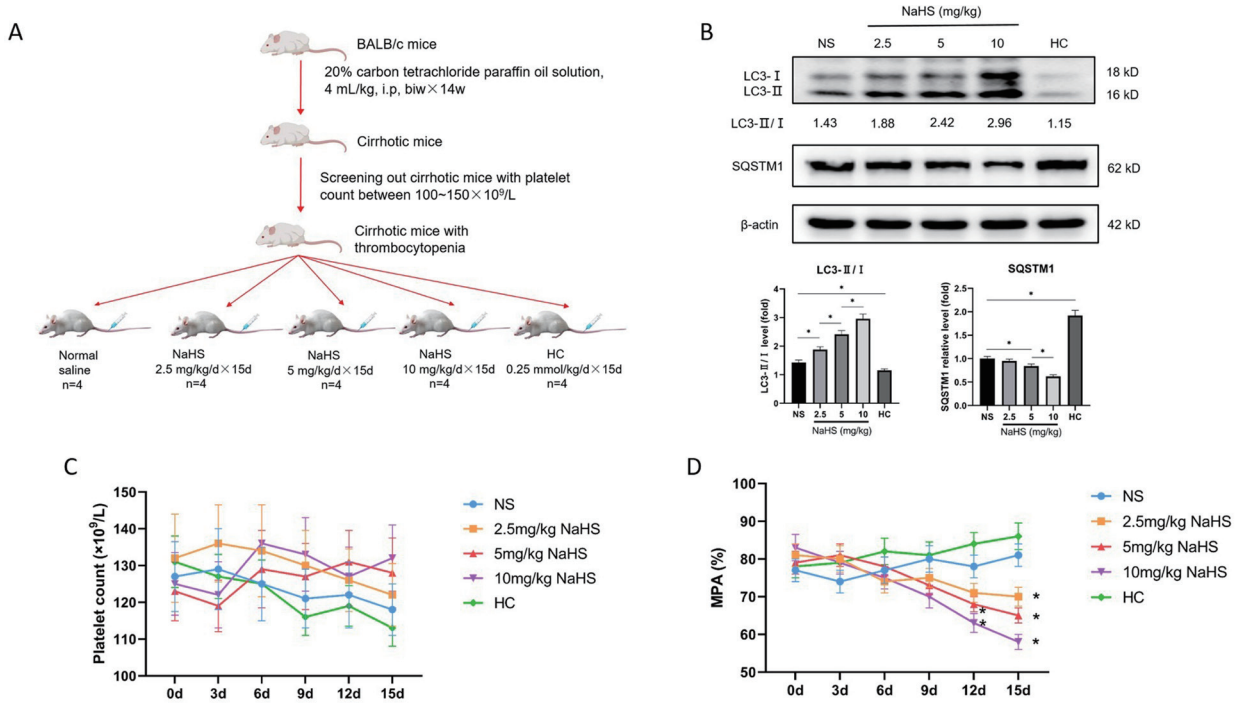

Fig. 6. H_2S promoted platelet autophagy and reduced platelet aggregation *in vivo*. (A) Establishment of mice model of cirrhotic thrombocytopenia. (B) NaHS injection increased the platelet LC3-II expression and reduced the SQSTM1 expression in a dose-dependent manner. After injection with hydroxocobalamin (HC, H_2S scavenger), the platelet LC3-II expression was significantly reduced, while the SQSTM1 expression was significantly increased. (C) After injection of different concentrations of NaHS or HC, there was no significant change in the platelet count of the mice at an indicated time. (D) After the injection of different concentrations of NaHS, the platelet aggregation rate significantly decreased on Day 15. * indicates $p<0.05$. i.p, intraperitoneal injection; biw, twice a week; NS, 0.9% normal saline; MPA, microscopic polyangiitis.

synthesis due to hepatocyte damage or excessive platelet destruction from hypersplenism.[2,16,17] However, the intracellular homeostasis of platelets is of great significance for maintaining their lifespan and function.[18,19] In recent years, it has been discovered that platelets can degrade damaged organelles through autophagy mechanisms to maintain intracellular homeostasis, which is similar to nucleated cells.[20–22] In 2014, Feng and colleagues first systematically elucidated the constitutive expression of the autophagy mechanism in human platelets, demonstrating its necessity to maintain the primary function.[7] Additionally, human platelets express components of the ULK1 complex, BECN1-PIK3C3 complex, and autophagosomes, confirming the existence of complete autophagic flux.[23]

This study revealed that platelet autophagy was significantly down-regulated in CTP and was dose-dependently augmented after H_2S stimulation. Enhanced platelet autophagy can reduce intracellular ROS levels, increase platelet viability, and inhibit apoptosis. Hence, impaired platelet autophagy can be a potential pathogenic mechanism in CTP. Consistent with our findings, Wang *et al*. observed diminished platelet autophagy in patients with immune thrombocytopenia, leading to increased platelet destruction through accelerated apoptosis.[24] Autophagy has a dual regulatory effect on cell apoptosis: mild autophagy protects cells from harmful stimuli, promoting survival, while extreme autophagy may lead to cellular dysfunction and induce apoptosis.[25–27] Damaged or degenerated subcellular components are eliminated through autophagy to prevent cell apoptosis. The degradation of damaged mitochondria inhibits cell apoptosis by preventing the release of pro-apoptotic molecules.[28,29] Depolarized mitochondria can be degraded through mitophagy, attenuating intracellular ROS levels and protecting cells from oxidative stress.[30] The down-regulation of mitophagy leads to DNA damage and chromosomal abnormalities in cells due to oxidative stress.[31] Enhancing platelet autophagy, as found in our study, reduced intracellular ROS levels, inhibiting platelet apoptosis. Additionally, during cellular energy crises, autophagy provides energy to cells by degrading organelles and proteins, prolonging cell lifespan.[32,33] We confirmed that up-regulating platelet autophagy with H_2S increased intracellular ATP levels in platelets through CTG assay, thereby enhancing platelet viability.

Moreover, we observed that H_2S could promote platelet autophagy and inhibit platelet aggregation both *in vivo* and *in vitro*. Previous studies confirmed that H_2S inhibited collagen-induced platelet aggregation via various signaling pathways and calcium ions in H_2S-induced platelets.[34,35] The PI3K signaling pathway, particularly in platelet autophagy, has garnered widespread attention. Promoting autophagy with PI3K inhibitors or inhibiting autophagic degradation with lysosomal inhibitor Baf A1 significantly affected platelet aggregation and adhesion.[36] Studies have confirmed that PI3K-dependent autophagy is necessary for maintaining essential platelet function.[7,37] Based on the above evidence, this study innovatively discovered that PDGFR was an upstream molecule of the platelet PI3K signaling pathway. H_2S downregulated the activation of the PI3K/Akt/mTOR signaling pathway by inhibiting PDGFR phosphorylation, thereby promoting platelet autophagy.

Additionally, another potential pathogenesis of CTP is excessive platelet aggregation, which prevents blood analyzers from recognizing platelets, resulting in significantly lower platelet counts than the actual value, a condition termed pseudothrombocytopenia.[38,39] This study revealed that platelet aggregation in cirrhotic patients was notably higher than in healthy controls, especially in those with moderate and severe thrombocytopenia. This finding may elucidate the thrombocytopenia observed in cirrhotic patients. Consistent with our results, previous research has confirmed an abnormal enhancement of platelet function (both aggregation and adhesion) in cirrhotic patients, irrespective of the thrombocytopenia status.[40–43] Interestingly, a recent study indicated that platelet function declined with worsening cirrhosis and portal hypertension.[44] However, this study solely employed flow cytometry to measure platelet activation as an indicator of platelet function and did not assess platelet aggregation and adhesion.

Nevertheless, this study had certain limitations. While we confirmed that H_2S influenced platelet apoptosis and function by regulating autophagy *in vitro*. In cirrhotic mice, we found that H_2S significantly affected platelet aggregation rather than platelet count. The discrepancy might be due to variations in baseline endogenous H_2S levels among cirrhotic mice, influenced by the catalytic activity of endogenous H_2S synthase such as cystathionine-β-synthase, cystathionine-γ-lyase, and 3-mercaptopyruvate sulfurtransferase.[45] Furthermore, the platelet count is influenced by the differentiation status of megakaryocytes. A recent study demonstrated that targeted knockout of ATG7 in hematopoietic stem cells inhibited autophagy, impairing megakaryocyte differentiation and reducing platelet production.[46] However, this study did not conduct further experiments to investigate the autophagy of megakaryocytes in cirrhotic mice due to limited experimental conditions.

Conclusion

Diminished platelet autophagy correlates with thrombocytopenia in cirrhotic patients. H_2S modulates platelet autophagy and functions possibly via the PDGFR-α/PI3K/Akt/mTOR signaling pathway.

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Conflict of interest

The authors have no conflicts of interest related to this publication.

Author contributions

Study concept and design (HXY, HGD), acquisition of data, drafting of the manuscript (HXY), analysis and interpretation of data (HXY, YJL, YLH, KKJ, LNL, HGD), critical revision of the manuscript for important intellectual content, administrative, technical, and material support (LNL, HGD), and study supervision (HGD). All authors have contributed significantly to this study and approved the final manuscript.

Ethical statement

This study was approved by the Ethics Committee of Beijing You'an Hospital (No.2020-066). All procedures in this study were conducted following the guidelines of the Declaration of Helsinki. All participants signed written informed consent for participation in this study.

Data sharing statement

The data of this study are available from the first author (HXY) upon the request.

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