

Heme oxygenase/carbon monoxide pathway inhibition plays a role in ameliorating fibrosis following splenectomy

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Abstract. Splenectomy is a recognized therapy for liver cirrhosis with splenomegaly, since it decreases free iron concentration that accompanies the destruction of red blood cells. Heme oxygenase (HO)-1 and its by-products, iron and carbon monoxide (CO), play crucial roles in hepatic fibrosis. The aim of the present study was to determine whether splenectomy in cirrhotic rats induced by bile duct ligation (BDL), through the HO/CO pathway, could slow down the development of liver fibrosis. Male Sprague-Dawley rats were divided randomly into the sham, BDL, splenectomy, Fe, zinc protoporphyrin (ZnPP) and cobalt protoporphyrin (CoPP) treatment groups, for inhibiting and inducing HO-1 expression. The level of HO-1 was detected by western blot analysis and reverse transcription-polymerase chain reaction. Serum carboxyhemoglobin (COHb), iron and portal vein pressure (PVP) were also quantified. Liver iron was measured by atomic absorption spectrometry with acetylene-air flame atomization. HO-1 and α -smooth muscle actin (α -SMA) were localized by immunohistochemistry. Liver and spleen iron were visualized by Perls' Prussian blue staining. Hepatic fibrosis was assessed using hematoxylin and eosin (H&E) staining. Enzyme-linked immunosorbent assay (ELISA) was used to detect serum transforming growth factor- β 1 (TGF- β 1). The results showed that liver, spleen and serum levels of HO-1, COHb and iron were greatly enhanced in the BDL group compared with the sham group; they were reduced following splenectomy and ZnPP treatment, but were elevated in the CoPP and Fe groups. Hydroxyproline, TGF- β 1, α -SMA, PVP and malonaldehyde levels were lower in the splenectomy and ZnPP groups compared to BDL, while higher levels were observed in the CoPP and Fe-treated groups. Our study shows that splenectomy reduces iron and CO levels in part by reducing HO-1 expression, and it decreases portal pressure and slightly decreases hepatic fibroproliferation.

Introduction

The spleen is connected to the other abdominal organs by the portal system. Portal hypertension and splenomegaly are often found in end-stage hepatic cirrhosis. As a treatment option, splenectomy is more widely accepted for patients without a donor suitable for liver transplantation (1). It is reported that splenectomy has beneficial effects on ameliorating reperfusion injury (2,3) and that it also induces a significant reduction in portal system pressure, improving hepatic function (4,5). Splenectomy also improves hepatic microcirculation, decreases spleen-derived endothelin-1, activates eNOS signaling, and inhibits the Rho-kinase pathway in rats with secondary biliary cirrhosis (6). Splenectomy also ameliorates liver injury in rats by mediating heme oxygenase-1 (HO-1) induction, thus yielding beneficial effects of massive hepatectomy and ischemia/reperfusion (7,8). The mechanism by which splenectomy influences cirrhotic liver remains unclear.

Previous studies, including our own, show that the HO/carbon monoxide (CO) pathway is involved in hepatic fibrosis and contributes to the hyperdynamic circulatory syndrome (9,10). HO-1 catalyses the oxidative degradation of heme to CO, free iron, and biliverdin (11). CO, a gaseous messenger similar to NO, leads to the production of cGMP, which mediates vasodilation (12). Our previous studies indicated that the HO/CO pathway may contribute to renal and pulmonary vasodilation in cirrhotic rats induced by bile duct ligation (BDL) (13,14). Whether splenectomy portal vein pressure (PVP) decreases are associated with a change in the HO/CO pathway is not clear.

Cirrhotic patients often have hypersplenism due to splenomegaly. Therefore, leukopenia, thrombocytopenia, and anemia are major concerns in cirrhotic patients (15). Damaged erythrocytes may lead to iron overload as each unit of erythrocytes contains approximately 250 mg of iron, thus increasing the risk of iron accumulation derived from increased heme degradation. Iron is an essential nutrient for growth and survival, but excess liver iron in particular induces oxidative stress and hepatic fibrogenesis (16). Iron is primarily accumulated in reticuloendothelial cells. The spleen also recycles iron stored in bone marrow and is essential for the removal of stored blood cells, and certain immune functions. Whether and how splenectomy influences iron homeostasis remains unclear.

Given the above-mentioned issues, the current study sought to evaluate how splenectomy impacts iron and CO levels, in

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part by reducing HO-1 expression, which further decreased PVP and reduced hepatic fibroproliferation.

Materials and methods

Animal care. The Animal Care and Use Committee of Dalian Medical University (Liaoning, China) approved the experimental protocols in accordance with guidelines established by the China Council on Animal Care.

Bile duct ligation and treatment of rats. The fifty healthy male SD rats, weighing 200–220 g, were obtained from the Laboratory Animal Center of Dalian Medical University and were randomly divided into six treatment groups: sham (n=6), BDL (n=10), cobalt protoporphyrin (CoPP) (n=10), zinc protoporphyrin (ZnPP) (n=8), iron-dextran (Fe) (n=8) and splenectomy (n=8). They were housed in a specific pathogen free (SPF) center at a room temperature of 24–26°C and relative humidity of 60–65%. Water was given *ad libitum*.

The rats were fed and housed for three days prior to any experimental protocols. Biliary cirrhosis was induced by BDL (17,18). Five groups underwent BDL together with a sham-operated animal group as a control. The Animal Care and Use Committee of Dalian Medical University approved all surgical procedures. Laparotomy was performed under anesthesia with ether. The bile duct was isolated and double-ligated with a 3-0 silk suture. The abdominal wall and skin were closed with a 4-0 silk suture, and antibiotic Gentamicin (0.3 ml) was injected intramuscularly. The sham group underwent laparotomy with the bile duct isolated but not ligated. Two weeks after surgery, sham and BDL rats received a saline intraperitoneal injection. For the splenectomy group, the abdomen was opened through a midline incision, and the spleen was immobilized to the centre of the operative field after dissecting the surrounding ligaments. The hilar vessels were ligated with a 3-0 silk suture. The spleen was removed and the abdominal incision was closed. Other groups received an intraperitoneal injection of CoPP, ZnPP, or iron-dextran (5, 5 and 50 mg/kg body weight), three times a week, respectively. Following establishment of the rat models, the number of rats was reduced to six in each group due to death in some groups.

ZnPP and CoPP (Sigma, St. Louis, MO, USA) were dissolved in 0.2 mol/l of NaOH, adjusted to pH 7.4 and diluted in 0.85% NaCl to 1 mg/ml as previously described, and used to inhibit and induce HO-1 expression. Fe (Sigma) was diluted in 0.85% NaCl to 20 mg/ml. Histostain™-Plus (SP9001) (Zhongshan Golden Bridge Biotechnology, Beijing, China), hydroxyproline (HYP), malonaldehyde (MDA) and glutathione (GSH) kits (Nanjing KeyGen Biotech Co. Ltd., China), transforming growth factor- β 1 (TGF- β 1) enzyme-linked immunosorbent assay (ELISA) kit (HopeYear Medical Products Co., Ltd., China), and Takara RNA polymerase chain reaction (PCR) kits (AMV) version 3.0 (Takara Bio, Inc., Dalian, China) were used in this study.

Sample collection and examination. Two weeks after treatment, a catheter connected to a Pressure Transducer (BL-420F biological experimental system; Chengdu Technology and Market Co., Ltd., China), was placed in the portal vein to measure PVP. Subsequently, 1 ml of arterial blood was withdrawn to measure

carboxyhemoglobin (COHb) using a RapidLab 1245 Blood Gas Analyzer (Siemens, New York, NY, USA). Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), and serum iron were detected using a Hitachi 7600-110 Automatic Biochemical Analyzer (Hitachi Co., Tokyo, Japan). Levels of MDA and GSH were determined by a UV-2100 Spectrophotometer (Chemito Instruments Pvt., Ltd., Mumbai, India).

Serum TGF- β 1. TGF- β 1 expression was determined by ELISA at 450 nm. Serum samples were assayed using 96-well microtiter plates coated with a polyclonal antibody against TGF- β 1.

Liver iron. Liver iron was determined by atomic absorption spectrometry with acetylene-air flame atomization. Analysis was performed using a Varian atomic absorption spectrometer (Mulgrave, Victoria, Australia) with a deuterium background correction. Measurements were performed with the analytical 248.3 nm line at a spectral interval of 0.2 nm. Iron concentration was determined by the standard addition method. Samples were digested in an MDS 2000 microwave sample preparation system (CEM) in Teflon cartridges using a mixture of nitric acid (5 ml) and hydrogen peroxide (2 ml) (ultrapure grade; both from Merck KGa, Darmstadt, Germany) for 20 min at the 120 psi. Resulting products were analyzed directly in the Teflon cartridges.

Histology and immunohistochemistry. Part of the liver and spleen lobe were excised, fixed in 10% neutral formalin solution, and embedded in paraffin. Hematoxylin and eosin (H&E) staining and Van Gieson's (VG) staining were performed according to standard procedures. Lesion severity was graded according to the methods described previously (19). Briefly, tissue sections (4 μ m) were treated with HCl (5%) to liberate ferric ions. Samples were then treated with 5% potassium ferrocyanide to produce insoluble ferric ferrocyanide. Slides were counterstained with neutral red. For immunohistochemical examination, deparaffinized sections were incubated with HO-1 antibodies (1:1,000 dilution; Abcam, Cambridge, MA, USA), or anti- α -smooth muscle actin (α -SMA) antibody (1:100 dilution; Boster Biological Technology Ltd., Wuhan, China) and biotinylated secondary antibodies, followed by avidin-biotin-peroxidase complex. Images were analyzed by Image-Pro-Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) to calculate area and mean density of positive expression. Statistical results from five visual fields were averaged from each sample.

Hepatic hydroxyproline content. Liver tissue (100 mg) was prepared for HYP determination according to a modification of the kit method described. The HYP content of the liver served as an indirect measure of tissue collagen content and was expressed as microgram/gram of wet weight (μ g/g).

Western blot analysis. The resected liver tissues were extracted with lysis buffer (1% Triton X-100; 50 mmol/l Tris-HCl, pH 7.6; 150 mmol/l NaCl; and 1% protease inhibitor cocktail). Western blot analysis protocols were previously described (20). Western blot analyses were performed with liver homogenates (30 μ g protein) using anti-HO-1 antibody (1:2,000 dilution;

Table I. Body weight and spleen weight in the sham and BDL groups at 2 weeks.

	2 weeks		
	Body weight (g)	Spleen weight (g)	Body weight (g)/Spleen weight (g) (%)
Sham	257±6	0.96±0.07	0.37±0.03
BDL	219±12 ^a	1.49±0.19 ^a	0.68±0.04 ^a

^aP<0.01 and ^bP<0.05 vs. sham group. BDL, bile duct ligation.

Table II. Serum biochemical index analysis in different groups (means ± SD, n=6/group).

	AST (U/L)	ALT (U/L)	TBIL (mg/dl)
Sham	139.00±4.52	36.83±1.72	0.83±0.23
BDL	255.83±7.55 ^a	49.83±4.36 ^a	11.90±1.96 ^a
CoPP	406.67±14.81 ^c	72.00±3.52 ^c	13.75±2.23 ^a
ZnPP	216.00±7.54 ^c	37.17±2.48 ^c	5.53±2.23 ^c
Fe	309.83±13.61 ^c	43.83±4.96 ^c	13.82±1.94 ^a
Splenectomy	202.17±6.85 ^{cf}	37.67±7.06 ^c	5.10±0.48 ^c

^aP<0.01 and ^bP<0.05 vs. sham group; ^cP<0.01 and ^dP<0.05 vs. BDL group; ^eP<0.01 and ^fP<0.05 vs. ZnPP group. BDL, bile duct ligation; AST, aspartate aminotransferase; TBIL, total bilirubin; CoPP, cobalt protoporphyrin; ZnPP, zinc protoporphyrin.

Abcam), anti-β-actin antibody (1:500 dilution; Zhongshan Golden Bridge Biotechnology), and secondary anti-rabbit and anti-mouse IgG (1:500 dilution; Biosynthesis Biotechnology, Beijing, China). The intensity of each signal was corrected by the values obtained from the immunodetection of β-actin and the relative protein intensity expressed as fold of the content in the control group.

RNA isolation and gene expression analysis. Total RNA was extracted from livers following a standard guanidinium phenol-chloroform extraction protocol. The quantity of RNA was determined by measuring the optical density at 260 nm (A260 nm = 1 for 40 μg/ml RNA), and RNA purity was assessed by determining the A260/A280 nm ratio (pure RNA: A260/A280 nm = 2.0) using a UV-1206 spectrophotometer (Shimadzu, Kyoto, Japan). An aliquot of each mixture was used for reverse transcription (RT)-PCR amplification using reagents purchased from Takara Bio, Inc. The primer sequences for HO-1 were 5'-ATATCTATACGGCCCTGGAA-3' (forward), 5'-GATGCTCGGGAAGGTGAA-3' (reverse) and the product size was 350 bp, while the primer sequences for β-actin were 5'-GAGGAAATCGTGCGTGAC-3' (forward), 5'-CTGGAAGGTGGACAGTGAG-3' (reverse) and the product size was 445 bp. PCR products were separated by 2.5% agarose gel electrophoresis. The product bands were photographed and the density of each band was quantified. The results are expressed as the ratio of the band density for the target mRNA to that of β-actin mRNA.

Statistical analysis. All data are presented as the means ± standard deviation. Statistical analysis was performed with SPSS

software version 16.0 (IBM, Chicago, IL, USA). Groups were compared using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test (where applicable). Correlative comparison of two non-hierarchical variances with normal distribution was evaluated by Pearson's test, whereas the Spearman test was used for data with a non-normal distribution. P-values <0.05 were considered to indicate statistically significant differences.

Results

Splenectomy ameliorates liver fibroproliferation and decreases PVP. The common bile duct dilation, ascites, and jaundice were found in all BDL rats at four weeks post-operation, suggesting that BDL was successfully established. Two weeks after the operation, the spleens were much bigger and heavier in the BDL group relative to the sham group (P<0.01), and the body weight (g)/spleen weight (g) ratio was larger in the BDL group (P<0.01) (Table I).

The serum levels of AST, ALT and TBIL in the BDL group were significantly higher than those in the sham group (P<0.01). Of note, they were at much lower levels in the splenectomy group than in the BDL group (P<0.01) (Table II). These data indicate that splenectomy improves liver function.

Hepatic fibrosis was evaluated by H&E staining. The sham group showed normal architecture (Fig. 1Aa), whereas the BDL group exhibited inflammation, necrosis, and destruction of the lobular architecture (Fig. 1Ab). Compared with the BDL group, less fibrous hyperplasia and fibrosis extensions with fibroblast proliferation were noted in the splenectomy group (Fig. 1Af). The histopathological scores for liver fibrosis were higher

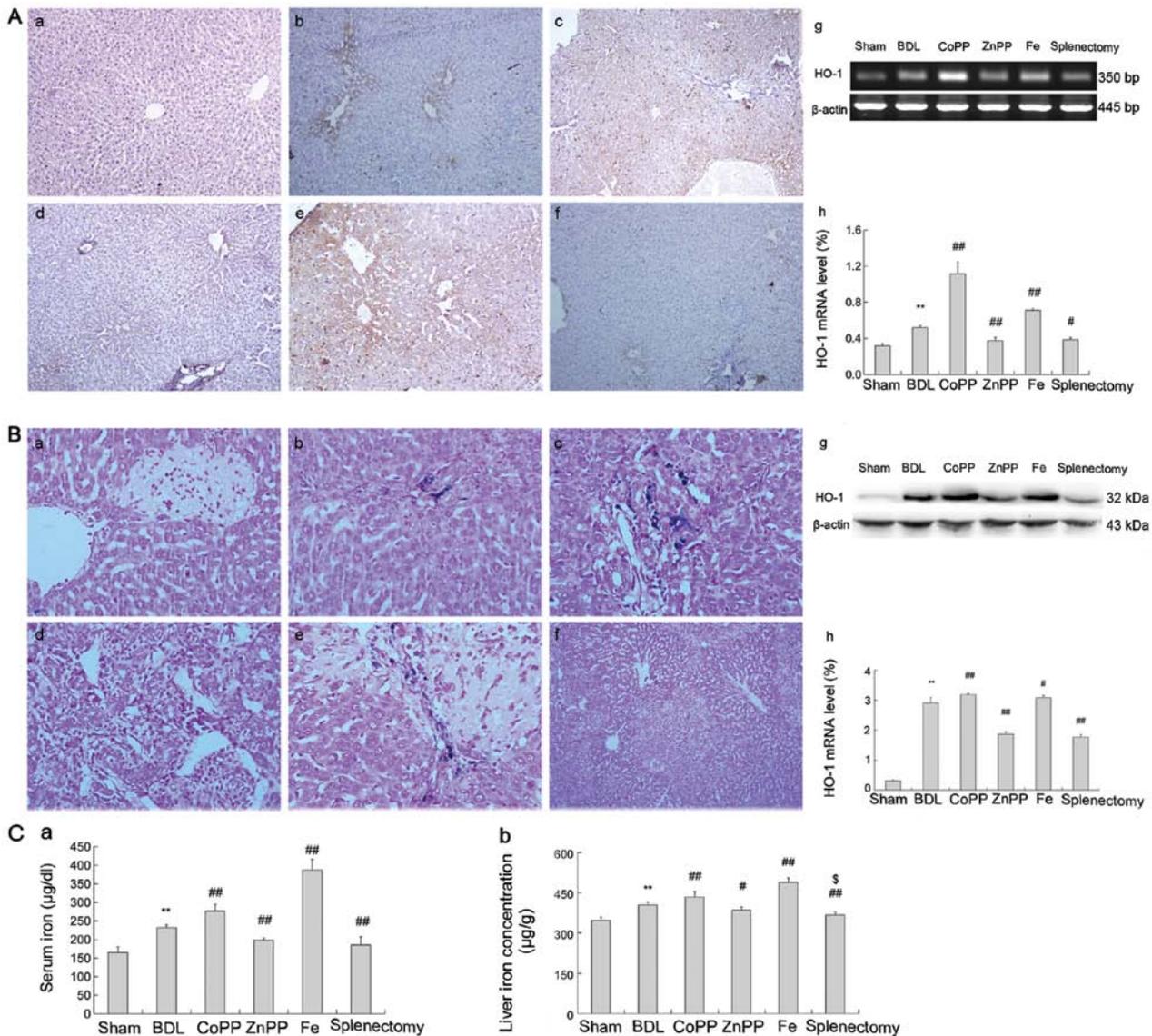


Figure 2. HO-1 and iron levels in liver. (A) Liver sections were stained with HO-1 antibody. Brown staining indicated immunopositivity. (a) HO-1 expression was minimal around central veins in the sham group. (b, c and e) HO-1 was observed around central veins in the BDL, CoPP and Fe groups. (d and f) There was reduced staining in the ZnPP and splenectomy groups. (g and h) HO-1 mRNA increased in the BDL group relative to sham and were enhanced in the CoPP and Fe groups relative to the BDL group, and decreased in the ZnPP and DFX groups (original magnification, x400). (B) Perls' Prussian blue staining positive for iron was observed as blue stain. (a) No iron accumulated in the sham group. (b) A little iron accumulated mainly on Kupffer cells in the BDL group. (c) Substantially more iron accumulation was found in interlobular and macrophagocytes in the CoPP group. (d and f) Slight iron accumulation was detected in the ZnPP and splenectomy groups. (e) Substantial iron accumulation was present in the Fe group. (g and h) HO-1 protein increased in the BDL group relative to sham and were enhanced in the CoPP and Fe groups relative to the BDL group, and decreased in the ZnPP and DFX groups. (C) Serum and liver iron content. Iron content in both liver and serum increased more in the BDL than in the sham group. (a and b) Iron content decreased in the ZnPP and splenectomy groups compared with BDL, and enhanced Fe was observed in the CoPP and Fe groups. The data are represented as the means \pm SD. ** $P < 0.01$, * $P < 0.05$ vs. sham; ## $P < 0.01$, # $P < 0.05$ vs. BDL; \$\$\$ $P < 0.01$, \$ $P < 0.05$ vs. ZnPP. a, Sham; b, BDL; c, CoPP; d, ZnPP; e, Fe; and f, splenectomy.

Splenectomy reduces iron accumulation by inhibiting HO-1 activity. The mRNA and protein expression levels of liver HO-1 increased significantly following BDL treatment compared to sham controls ($P < 0.01$); they were lower in the splenectomy and ZnPP groups ($P < 0.01$) than in the BDL group, but were elevated in the CoPP and Fe groups (Fig. 2Ag and h, Bg and h). Hepatic immunostaining showed that HO-1 was expressed mainly near centrilobular veins located on Kupffer cells, and partly in mesenchymal cells (Fig. 2Aa-f). Lower biochemical indicators, levels of α -SMA and TGF- β 1 were lower in ZnPP-treated rats than in those receiving BDL treatment, yet higher in the CoPP and Fe groups. These results suggest that

inhibiting HO-1 expression may improve liver function and decrease fibrosis. On the contrary, it aggravated liver injury (Fig. 1A and B).

Immunostaining of BDL spleen revealed multiple and patchy patterns of HO-1 (brown positive expression). Patterns were visible prominently in red pulp, while those in white pulp were few, if any (Fig. 3A-b). By contrast, the brown stain in the spleen collected from the CoPP and Fe-treated groups, and from macrophages in the red pulp, was markedly elevated. Cross-sections of red pulp appeared to be enlarged compared with analogous cross-sections from the BDL group, which decreased in splenectomy and ZnPP-treated rats (Fig. 3Aa-f).

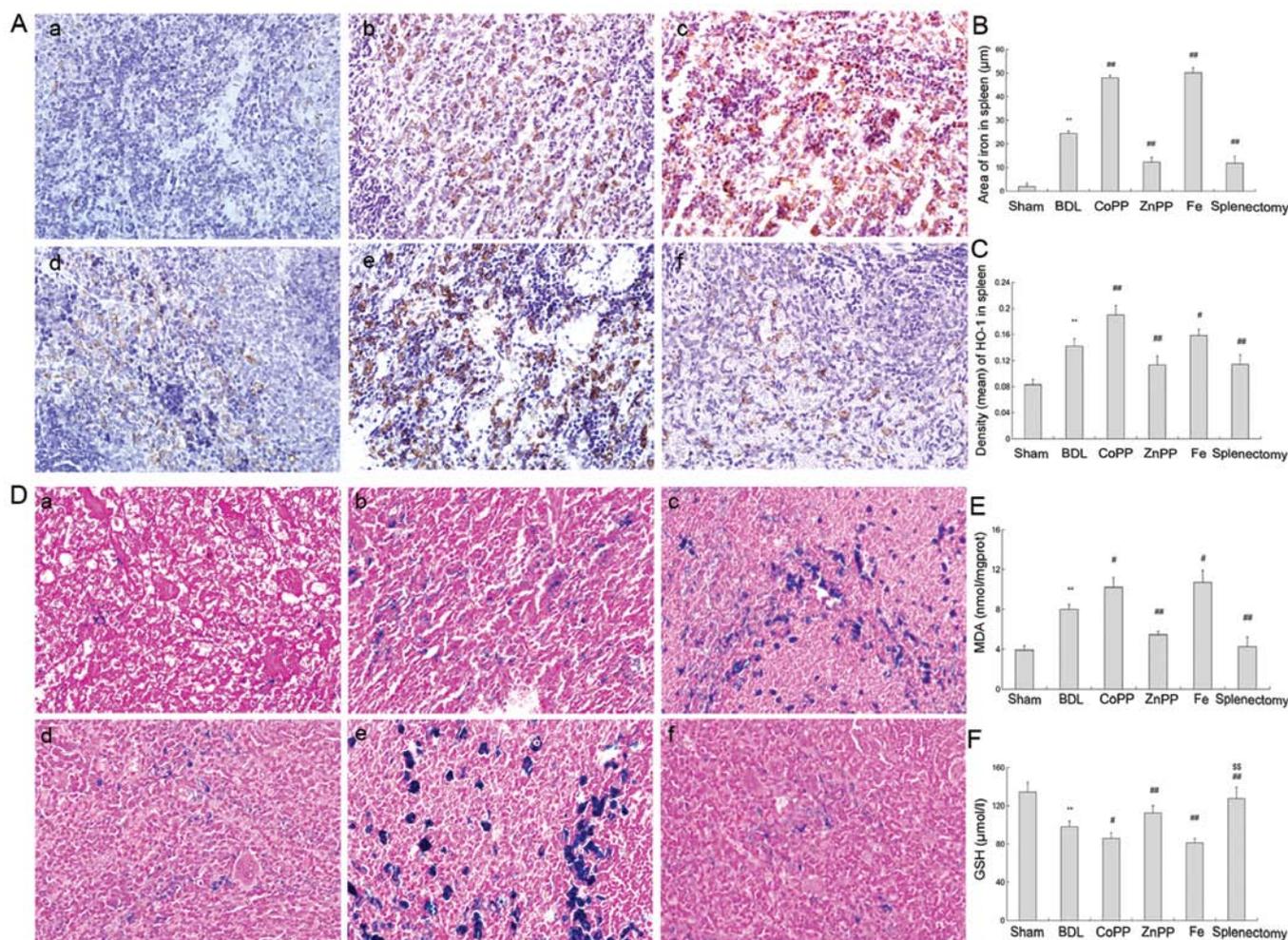


Figure 3. HO-1 and iron levels in the spleen. (A) Spleen sections were stained with HO-1 antibody. (a) Slight HO-1 expression was detected in the sham group. (b, c and e) There was more HO-1 expression in red pulp in the BDL, CoPP and Fe groups. (d and f) Compared with BDL rats, HO-1 expression decreased in the ZnPP and splenectomy groups. (B) Density (mean) of HO-1 in the spleen was measured by IPP, and was consistent with immunohistochemistry results ($P < 0.01$). (C) Area of iron in spleen was detected by IPP. The iron levels were higher in BDL than in sham rats. Iron levels were decreased in the ZnPP and splenectomy groups compared with BDL, but enhanced in the CoPP and Fe groups ($P < 0.01$). (D) Perls' Prussian blue staining in spleen. (a) Little iron accumulated in the sham group. (b, c and e) Iron accumulation was found in the red pulp in BDL, CoPP and Fe groups. (d and f) Compared with the BDL group, less iron was found in the ZnPP and splenectomy groups (original magnification, $\times 400$). (E) MDA levels in the liver were measured. MDA levels were markedly enhanced in BDL rats relative to sham rats. HO-1 was increased in the CoPP and Fe groups relative to the BDL group, but decreased in the ZnPP and splenectomy groups ($P < 0.01$). (F) GSH levels were measured in the liver, and moved in opposition to MDA levels. The data are represented as the means \pm SD. ** $P < 0.01$, * $P < 0.05$ vs. sham; ## $P < 0.01$, # $P < 0.05$ vs. BDL; \$\$\$ $P < 0.01$, \$\$ $P < 0.05$ vs. ZnPP. a, Sham; b, BDL; c, CoPP; d, ZnPP; e, Fe; and f, spleen from the splenectomy group at two weeks.

Changes in HO-1 protein expression levels in the spleen were similar to those in the liver (Fig. 3B).

The iron serum levels in the BDL group were significantly higher than in sham controls ($P < 0.01$), and were markedly lower following splenectomy and ZnPP treatments than after BDL treatment ($P < 0.01$) (Fig. 2Ca). Also, the change of liver iron content correlated with the change of serum iron levels (Fig. 2Cb). We used Prussian blue stain to localize iron accumulation in hepatic and splenic tissue. In the liver and spleen, iron accumulated in Kupffer cells and macrophages in the BDL, CoPP and Fe groups (Figs. 2B and 3D). However, iron staining was essentially absent in the sham, splenectomy, and ZnPP treated groups (Fig. 2Ba, d and f). The areas of iron accumulation in the spleen are shown in Fig. 3C.

These results indicate that the spleen plays an important role in HO-1 expression and iron accumulation, and that splenectomy reduces liver HO-1 expression and reduces iron

accumulation. By contrast, enhanced HO-1 expression led to increased accumulation of hepatic iron.

The HO/CO pathway is involved in regulating PVP. Splenic structure was evaluated by H&E staining (Fig. 1C). In normal spleen, macrophages were distributed in red pulp and marginal zones, but were less prevalent in BDL spleen (Fig. 1Ca-c). Collagen deposits were also detected in the spleen. More collagen fibers were found around the splenic corpuscle in the 4th week following BDL treatment than in the 2nd week (Fig. 1Da-c). This may be one factor regulating PVP formation in cirrhotic rats.

The COHb level in arterial blood was significantly higher in the BDL group than in the sham group ($P < 0.01$), significantly lower following splenectomy and ZnPP treatment, and higher in the CoPP and Fe-treated groups than in the BDL group ($P < 0.01$) (Fig. 1Dd). PVP was significantly higher in BDL than in sham

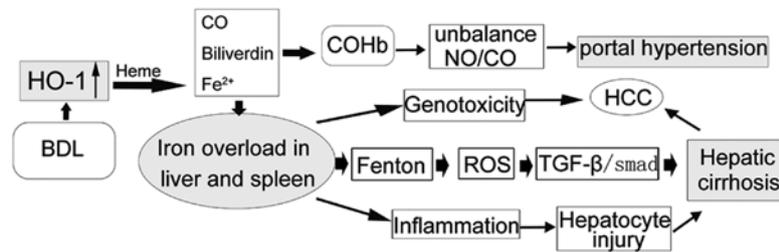


Figure 4. HO-1 regulates iron homeostasis and portal vein pressure in BDL rats. Inducing HO-1 expression mediates iron accumulation, which accelerates oxidative stress and further activates the TGFβ1/Smad pathway, aggravating liver cirrhosis. Excessive iron accumulation stimulates inflammation by the Fenton reaction and injures hepatocytes, leading to hepatocellular carcinoma due to increased genotoxicity. Splenectomy reduces iron overload and decreases PVP by inhibiting HO-1 activity, further alleviating liver cirrhosis.

($P < 0.01$). Compared with BDL, PVP decreased following splenectomy and ZnPP treatment ($P < 0.01$), and was enhanced in CoPP and Fe rats ($P < 0.01$) (Fig. 1Dd). Moreover, PVP decreased following splenectomy relative to ZnPP treatment ($P < 0.05$). We hypothesized that splenectomy may decrease COHb levels and regulate PVP through the HO/CO pathway.

Splenectomy reduces oxidative stress partly through the HO/CO pathway. MDA levels increased significantly following BDL compared to sham controls, and GSH levels were reduced ($P < 0.01$) (Fig. 3E and F). Splenectomy resulted in a lower level of MDA and elevated GSH levels relative to BDL ($P < 0.01$) (Fig. 3E). This suggests that oxidative stress was activated in cirrhotic rats.

The level of MDA was slightly higher in the CoPP and Fe-treated groups than in the BDL group ($P < 0.05$), but lower in ZnPP-treated rats. GSH levels were significantly enhanced by ZnPP treatment relative to BDL treatment ($P < 0.01$), but decreased following CoPP and Fe treatment (Fig. 3F). Moreover, GSH levels were elevated following splenectomy compared to ZnPP treatment ($P < 0.01$). Markedly, inhibiting HO-1 expression reduced oxidative stress, and induced HO-1 expression and iron accumulation, leading to liver injury.

Correlation analysis revealed that both SOD and MDA were significantly correlated with HYP levels ($R = -0.838$, 0.871 , respectively, $P < 0.01$). These data show that oxidative stress may lead to extracellular matrix (ECM) deposition in the liver and aggravate hepatic fibrosis. These data further indicate that reducing oxidative stress may lighten hepatic fibrosis, partly through the HO/CO pathway after splenectomy.

Discussion

Splenectomy is an effective operation for decreasing PVP. In this study, we found that splenectomy also influences the cirrhotic rat liver by altering HO-1 expression, resulting in iron homeostasis. Two weeks after splenectomy, the levels of AST, ALT and TBIL decreased, as did the serum concentrations of TGF-β1, α-SMA and HYP in the liver. Recent studies suggest that splenectomy may cause anti-inflammatory effects in the portal system in addition to decreasing PVP and reversing hypersplenism, which improves hepatic function (4,5). Moreover, splenectomy may moderate fibrosis by decreasing TGF-β1 secretion. Spleen-derived TGF-β1 plays an inhibitory role in healing hepatic cirrhosis by prohibiting regeneration

of damaged liver (21,22). We found it also improved liver function and decreased liver fibrosis following BDL, although splenectomy was initially performed to decrease PVP.

The majority of endogenous CO is catalyzed by inducible expression of HO-1. The release of CO by vascular cells may modulate blood flow and maintain the integrity of the vessel wall (23). It has been suggested that CO interacts with NO, which is a potent activator of soluble guanylate cyclase and a vasodilator (24,25). Indeed, excessive CO production, a consequence of HO-1 overexpression, could play a critical role in modulating vascular tone under different pathological situations (26). COHb levels can be used to estimate HO activity in experimental animals (27). Notably, we observed that upregulated COHb resulted from increased HO-1, which aggravated PVP in BDL rats. Moreover, splenectomy and lower levels of COHb can decrease PVP, with similar results found in the ZnPP treatment group. We reasoned that CO may decrease PVP in early stages, and excessive CO may be harmful by reducing PVP, leading to an unbalanced NO/CO system in the last stage of hepatic fibrosis. It therefore seems appropriate to reduce PVP by decreasing CO.

Our previous study indicated that HO-1 induction can ameliorate immune liver fibrosis (28). Upregulation of HO-1 interferes with chronic inflammation and prevents progression of liver fibrosis in Mdr2 knockout mice (29). Our study found that inhibition of HO-1 could modulate BDL-induced liver fibrosis, which was not in accordance with previous results. We hypothesized that HO-1 plays different roles in the progression of liver fibrosis. In early stage liver fibrosis, inducing HO-1 may have a protective action, but aggravates liver function and PVP during end stage cirrhosis with portal hypertension. Moreover, fibrosis resulting from different animal models may offer another explanation for these results.

We investigated the relationship between HO-1 expression and iron accumulation by inhibiting or inducing HO-1 expression. Inhibiting HO-1 activity reduced iron accumulation in the liver and spleen, and further attenuated hepatic fibrosis. Splenectomy produced similar results, which may be due, partly, to reduced HO-1 expression. Khan *et al* (30) reported that an increase in HO-1 expression is associated with iron accumulation, and HO-1 activity contributes to increased levels of intracellular labile iron (31). HO-1 gene expression is upregulated by iron, suggesting that degradation of internalized heme may be controlled by a positive feedback loop (32). In the present study, HO-1 was upregulated

following Fe treatment. However, anemia and iron accumulation in the kidney and liver are found in HO-1-deficient mice (33). Absent HO-1 and other HO subtypes, such as HO-2 and HO-3, may play a major role in iron homeostasis. We considered that HO-1 was necessary to form iron homeostasis, and inhibiting it, but not knocking it out, may be useful to reduce iron accumulation.

It has previously been shown that increased deposition of iron in the liver often triggers oxidative stress and inflammation and induces liver cell damage (34). Iron participates in the Fenton and Haber-Weiss reaction, and excessive redox-active iron leads to oxidative stress, with damage to membranes, proteins, and DNA (35). Nontransferrin-bound iron plays a key role in iron overload in severe cirrhosis (36). Our data show that iron accumulation increases oxidative stress and aggravates liver injury in CoPP and Fe-treated groups. This demonstrates that iron may play a pivotal role in hepatic fibrosis.

In our study, iron levels in serum, liver, and spleen all decreased in the splenectomy-treated animals, which showed lower HO-1 than the BDL group. Similar results were found in the ZnPP group. A previous study demonstrated that HO-1 activity is found in the spleen (37). We found enhanced HO-1 expression in the liver and spleen at two and four weeks following BDL. Spleen is a source of HO-1 production and splenectomy could therefore decrease HO-1 generation.

Previous studies have shown that spleen volume negatively correlates with red blood cell and platelet counting (38). Others have noted that splenic hypertrophy partly explains the anemia process, and splenomegaly is widely observed in human fascioliasis (39). These reports indicate that splenomegaly is a key reason for RBC damage and iron production from heme in cirrhotic patients. Splenectomy decreased red blood cell damage and further reduced iron production from heme degradation. This may be a benefit for cirrhotic patients with splenomegaly.

Reactive oxygen species (ROS) may represent a relevant profibrogenic stimulus for hepatic stellate cells (HSCs), promote production of type I collagen, and act as an intracellular signaling mediator of TGF- β (40,41). Splenectomy reduced oxidative stress in BDL rats and further decreased collagen deposition. Of note, however, similar results were found in the ZnPP group due to inhibition of HO-1 expression. HO-1 protected hepatocytes from ethanol-derived oxidative stress via the MAPK/Nrf2 pathway, in primary human hepatocytes (42). Iron accumulation increases liver injury through oxidative stress in nutritional steatohepatitis (43). We thus reasoned that the influences of pro-oxidant activities resulted from iron accumulation and were more effective than the anti-oxidant effects mediated by HO-1.

In conclusion, splenectomy decreases PVP. Moreover, we found it improves liver function partly through the HO/CO pathway during the hepatic cirrhotic process in rats. Inhibition of HO-1 expression by splenectomy led to reduced iron production (Fig. 4). However, we lacked a large-scale clinical trial and *in vitro* study to better clarify the exact role of the HO/CO pathway in cirrhotic patients following splenectomy, as it is difficult to obtain samples from patients who recover after surgery and who volunteer for a liver biopsy. In addition, we have reason to believe that a reduction in CO due to HO-1 inhibition may be a novel therapeutic option for decreasing PVP. The HO/CO

pathway may play a pivotal role in patients with splenectomy to intervene in liver cirrhosis and further reduce PVP.

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