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Original Article

Ascorbate lacks significant influence in rats with bile duct ligation-induced liver injury

Hsin-Ling Ho [a,d,e], Teh-Ia Huo [a,d], Ting Chang [b], Wen-Shin Lee [b,e], I-Fang Hsin [c,d,e], Fa-Yauh Lee [a,e], Hui-Chun Huang [a,b,e,*], Ming-Chih Hou [a,e], Shou-Dong Lee [e,f]

[a] *Division of Gastroenterology and Hepatology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*
[b] *Division of General Medicine, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*
[c] *Division of Endoscopy Center for Diagnosis and Treatment, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*
[d] *Department and Institute of Pharmacology, National Yang-Ming University School of Medicine, Taipei, Taiwan, ROC*
[e] *Faculty of Medicine, National Yang-Ming University School of Medicine, Taipei, Taiwan, ROC*
[f] *Division of Gastroenterology, Department of Medicine, Cheng Hsin General Hospital, Taipei, Taiwan, ROC*

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Abstract

Background: Liver inflammation may induce fibrogenesis, cirrhosis and portal hypertension. Liver cirrhosis is characterized by increased intrahepatic resistance and enhanced vasoconstrictive response. The splanchnic vasodilatation, angiogenesis and portosystemic collaterals formation further bring about lethal complications. Ascorbate is a potent antioxidant with anti-inflammation, anti-fibrosis, and anti-angiogenesis effects. However, the relevant influences in chronic liver injury have not been sufficiently explored.
Methods: Chronic liver injury was induced in Spraque-Dawley rats with common bile duct ligation (BDL). Ascorbate (250 mg/kg/day, oral gavage) or vehicle was administered starting on the 1st day after operation. On the 8th (hepatitis) and 29th (cirrhosis) day, serum biochemistry parameters, hepatic concentrations of lipid peroxidation-related substances, protein expressions of α-SMA, TGF-β, iNOS, eNOS, p-eNOS-Ser1177, p-eNOS-Thr496, VEGF, VEGFR2, p-VEGFR2, and liver histology were evaluated. In three series of paralleled groups, rats treated with 28-day ascorbate or vehicle received hemodynamic measurements, hepatic and collateral vasoresponsiveness perfusion experiments, mesenteric CD31 immunofluorescence staining, and Western blot analyses of mesenteric VEGF, VEGFR2, pVEGFR2, PDGF, PDGFβ, COX1, COX2, eNOS, p-eNOS-Thr495, p-eNOS-Ser1177 protein expressions. In another series, the severity of portosystemic shunting was evaluated.
Results: Ascorbate did not influence hepatitis, oxidative stress, fibrosis, and hemodynamic parameters in BDL rats. The intrahepatic and collateral vasoresponsiveness were not affected, either from direct incubation or acute treatment with ascorbate. Furthermore, the mesenteric angiogenesis and severity of shunting were not influenced.
Conclusion: The oxidative stress, fibrosis, hemodynamic derangements, angiogenesis and vascular functional changes in BDL-induced chronic liver injury may be too overwhelming to be modulated by ascorbate.

Keywords: Ascorbate; Hepatitis; Liver cirrhosis; Portal hypertension

1. Introduction

Chronic liver injury may elicit a cascade of fibrogenesis and ultimately, liver cirrhosis and portal hypertension. Liver cirrhosis is characterized by increased intrahepatic resistance owing to fibrotic bands and regeneration nodules (structural component) and enhanced intrahepatic vasoconstrictive

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* Corresponding author: Dr. Hui-Chun Huang, Division of General Medicine, Department of Medicine, Taipei Veterans General Hospital, 201, Section 2, Shi-Pai Road, Taipei 112, Taiwan, ROC.
E-mail address: hchuang2@vghtpe.gov.tw (H.-C. Huang).

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response (functional component). The insufficient intrahepatic bioavailability of vasodilators such as nitric oxide (NO) is also noted.[1] On the other hand, the peripheral and splanchnic vasodilatation lead to decreased systemic vascular resistance, compensatorily increased heart rate and cardiac output, increased splanchnic blood flow, portal inflow and portal pressure (PP). The difficulty of the portal blood flow in entering the liver induces formation of portosystemic collaterals,[2] in which angiogenesis also plays a role.[3] Recent studies have revealed that vascular endothelial growth factor (VEGF) is overexpressed in splanchnic organs from portal hypertensive rats. Furthermore, angiogenesis triggers the development and maintenance of splanchnic hyperemia and portosystemic collaterals, which can be ameliorated by VEGFR2 (VEGF receptor 2) blockade.[4,5] These derangements, actually, are responsible for dreadful complications such as gastroesophageal variceal hemorrhage.

Indeed, apart from managing complications of liver cirrhosis and portal hypertension, the control of hepatic injury at the beginning is pivotal, and the substances easily obtained and inexpensive without significant toxicity are ideal candidates. It has been identified that oxidative stress with lipid peroxidation is a common pathological mechanism contributing to the initiation and progression of hepatic damage.[6] Upon inflammation, lipid peroxidation products recruit neutrophil and aggravates liver injury.[7] They also launch fibrogenesis by enhancing fibrogenic gene expression in activated stellate cells.[8,9] Therefore, antioxidant administration is a potentially feasible therapeutic strategy to delay the process of liver damage.[10]

Among the food-derived antioxidants, ascorbate (ascorbic acid, vitamin C) may be the most well-known and prevalent. Ascorbate provides *in vivo* antioxidant protection primarily as an aqueous phase peroxyl and oxygen radical scavenger. It is concentrated in tissues with a high potential for free radical generation, such as the eye, brain, liver, lung, and heart. Some evidence indicates that ascorbate protects against lipid peroxidation by regenerating the reduced form of α-tocopherol, the primary lipid-phase antioxidant.[11,12]

Regarding the levels of ascorbate in patients with chronic liver diseases, it has been reported that antioxidant vitamin levels, especially vitamin C, were significantly lower in patients with primary biliary cirrhosis.[13] In another study, a significant decrease in plasma ascorbate level was observed in patients with chronic active hepatitis and liver cirrhosis,[14] indicating that these patients were subject to oxidative stress.

Ascorbate has also aroused much attention due to its beneficial vascular effects. Ascorbate reversed NO-dependent endothelial dysfunction in coronary or peripheral arteries of patients with atherosclerosis.[15] Heitzer et al.[16] measured forearm microvascular responses to acetylcholine in long-term smokers, and observed a marked improvement in acetylcholine-induced vasodilation with intra-arterial high dose infusion of vitamin C. Regensteinera et al. also found that ascorbate improved flow-mediated brachial artery dilation (FMD) in patients with type 2 DM.[17] Similarly, Motoyama et al. observed a significant short-term improvement in brachial artery endothelial function after acute parenteral infusion of vitamin C in smokers. At the same time, a significant decrease was seen in the plasma levels of thiobarbituric acid–reactive substances, suggesting an acute reduction in oxidative stress.[18]

The vascular effect of ascorbate may be mediated via the modulation of NO activities. L-ascorbic acid potentiates agonist-induced endothelial NO synthesis.[19,20] Decreased endothelial NO activity is a common feature of type 2 diabetes and may contribute to the development of vascular complications, which can be improved by intra-arterial administration of vitamin C.[21] Ascorbate also potentiates the tetrahydrobiopterin-dependent endothelial NOS (eNOS) activation via enhancing the affinity of tetrahydrobiopterin for eNOS.[22] Regarding chronic treatment, Hornig et al.[23] observed a significant improvement in radial artery FMD in patients with chronic heart failure after four weeks of high dose oral vitamin C supplementation (1 g twice daily).[24] Recently, Gokce et al. reported a sustained beneficial effect on endothelial function after one-month treatment with ascorbate.[25]

It is worth noting that ascorbate inhibits angiogenesis as well.[26] The level of VEGF was significantly higher in mice challenged with melanoma B16FO cells than in ascorbate-supplemented mice.[27] A previous study indicated a significant inverse correlation between ascorbate and VEGF protein levels.[28] It has also been found that L:-ascorbate inhibited the secretion of vascular VEGF and the growth of xenograft pancreatic tumor in athymic mice.[29]

The distinct features of ascorbate may be beneficial for various stages of liver injury, but the relevant survey has not yet been performed. In addition, ascorbate is water-soluble without significant toxicity, which makes it feasible for patients with liver diseases. This study, therefore, aimed to investigate if ascorbate modulates the aforementioned aspects in rats with common bile duct ligation (BDL)-induced chronic liver injury.

2. Methods

2.1. Animal model: common bile duct ligation (BDL)

Bile duct ligation is a well-established animal model to induce liver injury via cholestasis. Typically, liver inflammation and cirrhosis develop beginning on the 5th and 28th day after BDL,[30,31] respectively, which makes this model appropriate for the survey of various stages of liver injury. In this study, male Sprague–Dawley rats weighing 240–270 g at the time of surgery were used. BDL was performed as previously described[32] under ketamine anesthesia (100 mg/kg, intramuscularly). To avoid the coagulation defects, weekly vitamin K injection (50 μg/kg intramuscularly) was applied.[30] All the procedures were conducted in accordance with the principles of laboratory animal care [Guide for the Care and Use of Laboratory Animals, DHEW publication No. (NIH) 85-23, rev. 985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD., USA.]. The Taipei Veterans General Hospital

Animal Committee approved the study (IACUC 2014-103, IACUC 2014-106, IACUC 2014-107).

2.2. Measurement of systemic and portal haemodynamics

Mean arterial pressure (MAP), heart rate (HR) and portal pressure (PP) were measured by catheterization of the right femoral artery and ileocolic vein, respectively. Pressure was transmitted through a Spectramed DTX transducer (Spectramed Inc., Oxnard, CA, USA). Continuous recordings were performed on a multichannel recorder (model RS 3400, Gould Inc., Cupertino, CA, USA).[33] To measure the superior mesenteric artery blood flow (SMA flow), SMA was identified at its aortic origin and a 5-mm segment was gently dissected free from surrounding tissues. Then a pulsed-Doppler flow transducer (T206 small animal blood flow meter, Transonic Systems Inc., Ithaca, NY, USA) was gently placed.[34] Cardiac output (CO) was measured by thermodilution, as previously described.[35] Briefly, the aortic thermistor was connected to a Columbus Instruments Cardiotherm 500-AC-R (Columbus Instruments International Co., Columbus, OH, USA). Five thermodilution curves were obtained for each cardiac output measurement. The final cardiac output value was obtained from the arithmetic mean of the results. Cardiac index (CI, ml/min/100 g BW) was calculated as cardiac output per 100 g BW. Systemic vascular resistance (SVR, mmHg/ml/min/100 g BW) was calculated from dividing MAP by CI. SMA resistance (SMAR, mmHg/ml/min/100 g BW) was calculated by (MAP-PP)/SMA flow per 100 g BW.

2.3. Determinations of hepatic oxidative stress parameters

The livers were pulverized with liquid nitrogen. The powder was diluted with ice-cold PBS (pH 7.4) without heparin at a ratio of 1:9 (w/v) and then centrifuged (10,000 g, 4 °C, 15 min). Hepatic concentrations of lipid peroxidation-related substances: superoxide dismutase (SOD), malondialdehyde (MDA), trolox equivalent antioxidant capacity (TEAC), catalase, and glutathione peroxidase were measured as previously described.[36]

2.4. In situ perfusion of liver for the hepatic vascular response study

The *in situ* liver perfusion technique is modified from that reported by Mittal et al.[37] with a non-recirculating setting. Briefly, the bile duct was cannulated with a polyethylene PE-10 tube, and the hepatic artery was ligated. A ligature was passed around the inferior vena cava (IVC) above the renal veins and the IVC injected with 500 U of heparin. The portal vein was then cannulated with a 16-gauge Teflon catheter, then the animal was transferred into a warm chamber (37 ± 0.5 °C). The liver was immediately perfused with Krebs solution via the portal vein cannula with a constant flow rate of 40 ml/min by a roller pump (model 505S, Watson-Marlow Limited, Falmouth, Cornwall, UK). The perfusate was equilibrated with carbogen gas (95% O2-5% CO2) by a silastic membrane lung.[38] The IVC was then cut below the ligature, thus allowing the perfusate to escape. Thereafter, the thorax was opened and the supradiaphragmatic part of IVC was cut to ensure an adequate outflow without any resistance. The ligature around the infrarenal IVC was subsequently tied. All the experiments were performed 30 min after starting perfusion when the system was stabilized.

2.5. In situ perfusion of portosystemic collaterals

The *in situ* perfusion system was performed as previously described.[39] Both jugular veins were cannulated with 16-gauge Teflon cannulas to ensure an adequate outflow without any resistance. Heparin (200 u/100 g) was injected through one of the cannulas. The 18-gauge Teflon cannula inserted into the distal mesenteric vein served as the inlet. To exclude the liver from perfusion, the second loose ligature around the portal vein was tightened. The animal was then transferred into a warm chamber (37± 0.5 °C). A Spectramed DTX transducer attached to the Gould model RS 3400 recorder was used to continuously monitor the pressure of the collateral vascular bed. All the experiments were performed 25 min after starting perfusion at a constant rate of 12 ml/min. In each individual preparation, the contracting capability of the collaterals was challenged with a 125-mM potassium chloride solution at the end of experiments.

2.6. Western blot

The liver and mesentery were extracted by pulverization with liquid nitrogen, then a ratio of 1 ml of lysis buffer (Roche Diagnostics GmbH, Penzberg, Germany) for each 100 mg powdered liver sample was used. Protein concentration was determined for each sample by the Bradford method.[40] An aliquot of 20–40 μg protein from each sample and 10 μg positive control were separated using denaturing SDS-10% polyacrylamide gels by electrophoresis (Mini-PROTEAN® 3 Cell, Bio-Rad Laboratories, Hercules, CA, U.S.A.). Prestained proteins markers (SDS-PAGE Standards, Bio-Rad) were used for molecular weight determinations. Proteins were then transferred to a polyvinylidene difluoride membrane (Immum-BlotTM PVDF Membrane, Bio-Rad) by a semi-dry electroblotting system (Trans-Blot® SD Semi-dry Electrophoretic Transfer Cell, Bio-Rad) for 1.5 h at 4 °C. To block non-specific binding, membranes were blocked for 30 min with 3% non-fat dry milk in TBS-T, pH 7.4. Blots were incubated with the primary and second antibodies, respectively. Detection of the specific proteins was performed by enhanced chemiluminescence (BCIP/NBT solution, Amresco Co., Solon, OH, USA). With a computer assisted video densitometer and digitalized software (Kodak Digital Science TM ID Image Analysis Software, Eastman Kodak Co., Rochester, NY, USA), the blots were scanned, photographed, and then the signal intensity (integral volume) of the appropriate band was analyzed.

2.7. Immunofluorescent study for the mesenteric vascular density

Mesenteric angiogenesis was quantified by CD31-labelled microvascular networks in rat mesenteric connective tissue windows according to the previous study.[34] At least four sets of data were obtained for each mesenteric window. (×100)-magnification immunofluorescent images were assessed using an upright fluorescent microscope (AX80, Olympus, Japan) with a charge-couple device (QICAM, High-performance IEEE 1394 FireWireTM Digital CCD Camera, Q IMAGING, BD - Canada) and thresholded by Image J software (National Institutes of Health (http://rsb.info.nih.gov/ij/)). The vascular length was manually measured with the pencil tool, and the vascular area was automatically measured with histogram function, respectively. According to the information provided by QICAM, with the eyepiece 10×, the diameter of one pixel on an image taken with QICAM equals 4.65 μm. Under 100×-magnification (objective 10× and eyepiece 10×), the diameter of one pixel will be 4.65 μm/10 = 0.465 μm. The vascular length was thus determined accordingly. The unit of vascular length per unit area of mesenteric window is $\mu m \cdot (\mu m^2)$-1 = μm-1, and the vascular area per unit area of mesenteric window, actually, can be pixel·pixel-1 without being converted to $\mu m^2 \cdot (\mu m^2)^{-1}$.

2.8. Color microsphere method for portosystemic shunting degree analysis

Portosystemic shunting degree was determined according to the technique introduced by Chojkier,[41] except for substitution of color for radioactive microspheres. In brief, 30,000. Fifteen micrometre yellow microspheres (Dye Track; Triton Technology, San Diego, CA, USA) were slowly injected into the spleen. The rats were then euthanized, thereafter the livers and lungs were dissected. The number of microspheres in each tissue was determined following the protocol provided by the manufacturer. There were 3000 blue microspheres that served as the internal control. Any spillover between wavelengths was corrected using the matrix inversion technique. The portosystemic shunting was calculated as follows: (number of microspheres) lung/(liver + lung). Assuming a worst-case scenario in which two-thirds of the microspheres remain trapped in the spleen, this technique detects a minimal shunt of 3.5%. Studies using color microspheres have been validated to provide results similar to those using radioactive microspheres.[42]

2.9. Hepatic fibrosis determination with sirius red staining

Liver paraffin section was stained with Sirius red staining kit (Polysciences Inc., Warrington, PA, USA), and Image J was used to measure the percentage of Sirius red-stained area.[34]

2.10. Hydroxyproline content assay

In brief, three small fragments of each liver were pooled, homogenized in distilled water, and lyophilized, and 20 mg of the freeze-dried sample was hydrolyzed at 95 °C for 20 min. After hydrolysis, the samples were neutralized at pH 6.0 to 6.8. The hydrolysates were then treated with activated charcoal. After centrifugation at 1000 rpm for 10 min, aliquots of the hydrolysates were used to measure hydroxyproline content spectrophotometrically by reaction with Ehrlich's reagent. Absorbance was measured at 560 nm.

2.11. Determinations of liver biochemistry parameters

Serum ammonia, alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine were performed by colorimetric assay (Daiichi AH 600 analyser; Olympus Co., Tokyo, Japan).

2.12. Drugs

Ascorbate, endothelin-1 (ET-1) and arginine vasopressin (AVP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solutions were freshly prepared on the days when experiments occurred.

2.13. Statistical analysis

All results are expressed as mean ± SEM. Statistical analyses were performed using an unpaired Student's *t*-test. Results were considered statistically significant at a two-tailed P-value less than 0.05.

2.14. Study protocol

(1) Liver inflammation: the 8th day after BDL

Ascorbate 250 mg/kg/day (oral gavage) or vehicle was administered beginning on the 1st day after BDL. The following parameters were evaluated on the 8th day, a typical stage of BDL-induced liver inflammation[30,31]: (a) serum levels of ALT, AST, BUN, and creatinine; (b) hepatic concentrations of lipid peroxidation-related substances: SOD, MDA, TEAC, catalase, glutathione peroxidase.

(2) Liver cirrhosis: the 29th day after BDL

On the 29th day, a typical stage of BDL-induced liver cirrhosis,[30,31] experiments were performed in BDL rats with 28-day vehicle or ascorbate treatment to evaluate: (a) systemic and portal hemodynamic parameters; (b) hepatic protein expressions ofα-SMA, TGF-β, iNOS, eNOS, p-eNOS-Ser1177, p-eNOS-Thr496, VEGF, VEGFR2, p-VEGFR2; (c) liver histology (Sirius red staining); (d) hepatic hydroxyproline content.

(3) Hepatic and collateral vasoresponsiveness study on the 29th day after BDL

Hepatic vasoresponsiveness was evaluated in two aspects: (i) direct incubation: the *in situ* liver perfusion model was applied for BDL rats preincubated with ascorbate (10^{-4} M) or vehicle. Sixty minutes later, the intrahepatic vascular responsiveness to endothelin-1 (10^{-10}, 10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8} M) was evaluated. To avoid light-induced ascorbate oxidation, the equipment for perfusion experiments, especially tubes and containers, were covered or wrapped with aluminum foil; (ii) Acute treatment effect: BDL rats received ascorbate (100 mg/kg, i.v.) or vehicle injection. 60 min later, the intrahepatic ET-1 vasoresponsiveness was evaluated. Collateral vasoresponsiveness was evaluated with the same study design as (b), except that AVP (10^{-10}, 10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7} M) instead of ET-1 was applied.

(4) Shunting and mesenteric angiogenesis

In BDL rats with 28-day vehicle or ascorbate treatment, the severity of shunting was evaluated using the color microsphere method. In paralleled groups, the following were assessed: (a) mesenteric vascular density with CD31 immunofluorescent study; (b) mesenteric angiogenic protein expressions of VEGF, VEGFR2, pVEGFR2, PDGF, PDGFβ, COX1, COX2, eNOS, p-eNOS-Thr495, p-eNOS-Ser1177.

The doses of ascorbate with different administration routes were decided based upon the previous literature.[26,43–46]

3. Results

3.1. Liver inflammation: the 8th day after BDL

3.1.1. Liver and renal biochemistry parameters

Fig. 1 reveals that there was no significant difference in AST (vehicle vs. ascorbate: 1118.5 ± 416.5 vs. 787.8 ± 67.2 U/L), ALT (186.6 ± 86.4 vs. 106.8 ± 20.8 U/L), BUN (22.0 ± 3.3 vs. 21.8 ± 0.9 mg/dL) and creatinine (0.19 ± 0.01 vs. 0.18 ± 0.01 mg/dL) between BDL rats with 7-day vehicle (n = 8) or ascorbate (n = 8) treatment (all $p > 0.05$).

3.1.2. Oxidative stress parameters

The oxidative stress parameters in BDL rats with 7-day vehicle (n = 8) or ascorbate (n = 8) treatment are shown in Fig. 2. There was no significant difference in SOD (1.82 ± 0.22 vs. 2.06 ± 0.39 unit/mg protein), MDA (2.59 ± 0.50 vs. 2.65 ± 0.60 nmol/mg protein), TEAC (68.21 ± 4.28 vs. 66.83 ± 3.78 μmol/mg protein), catalase (0.95 ± 0.14 vs. 0.96 ± 0.07 U/mg protein), and glutathione peroxidase (1886.82 ± 146.02 vs. 2040.54 ± 296.18 U/mg protein) (all $p > 0.05$).

3.2. Liver cirrhosis: the 29th day after BDL

3.2.1. Body weight and hemodynamics

There was no significant difference in body weight (BW, 339.1 ± 18.4 vs. 336.5 ± 10.2 g), SMA flow (5.8 ± 0.5 vs. 6.7 ± 0.6 ml/min/100 g), PV flow (5.3 ± 0.7 vs.

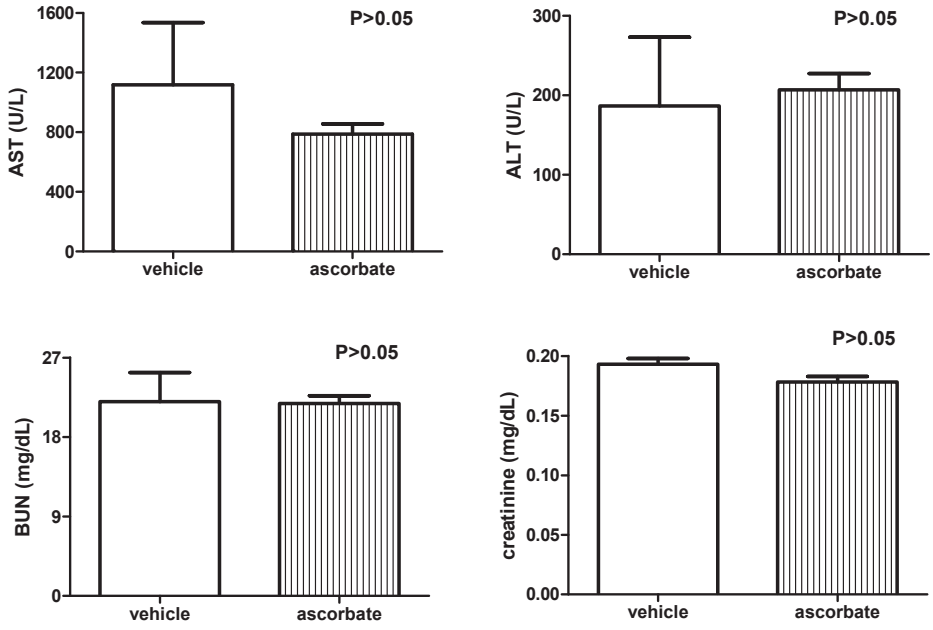

Fig. 1. Liver and renal biochemistry parameters in BDL rats with 8-day vehicle or ascorbate treatment. There was no significant difference in alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine (Cr) (all $p > 0.05$). The representative hepatic H&E staining images are shown below.

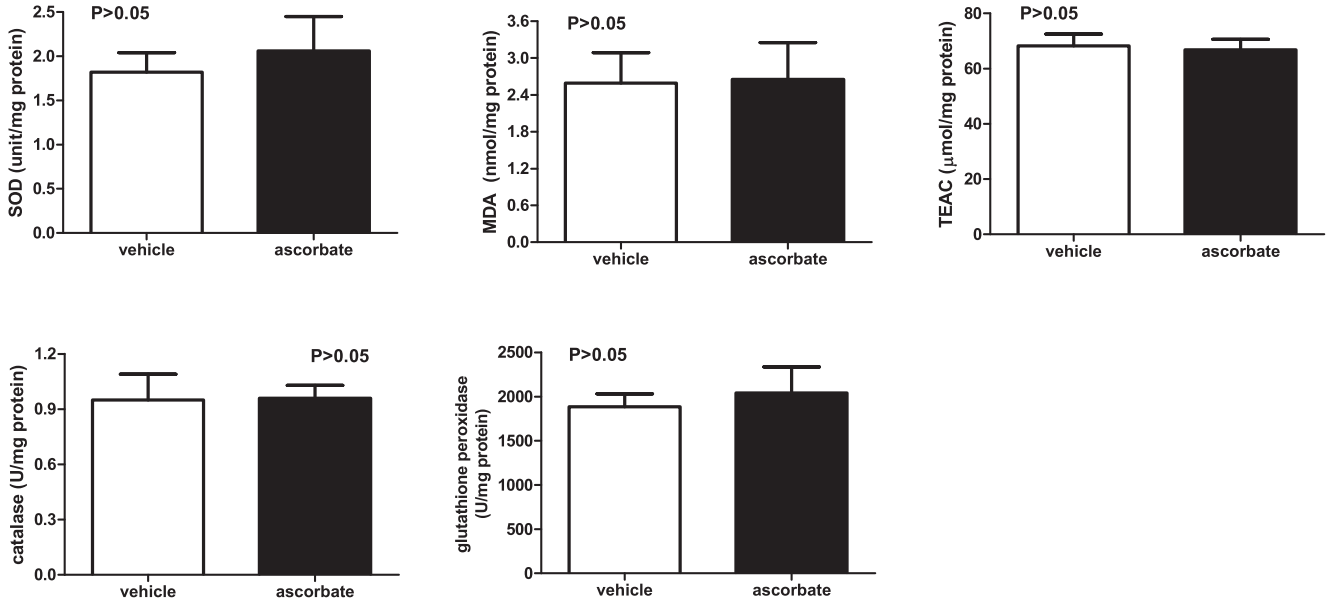

Fig. 2. The oxidative stress parameters in BDL rats with 8-day vehicle or ascorbate treatment. There was no significant difference in superoxide dismutase (SOD), malondialdehyde (MDA), trolox equivalent antioxidant capacity (TEAC), catalase, and glutathione peroxidase (all $p > 0.05$).

6.0 ± 0.7 ml/min/100 g), MAP (118 ± 4 vs.115 ± 2 mmHg), PP (14.7 ± 0.7 vs. 16.9 ± 1.2 mmHg) and CI (28.06 ± 3.25 vs. 39.37 ± 5.16 ml/min/100 g) (all $p > 0.05$) between BDL rats with 28-day vehicle (n = 8) or ascorbate (n = 8) treatment.

3.2.2. Hepatic western analyses

The hepatic α-SMA (/β-actin: 1.8386 ± 0.4237 vs. 1.7933 ± 0.3818), TGF-β (1.4226 ± 0.2461 vs.1.3128 ± 0.2495), iNOS (0.9570 ± 0.4793 vs. 1.0639 ± 0.1005), eNOS (0.7521 ± 0.5415 vs. 0.8229 ± 0.1021), p-eNOS-Ser1177 (1.0389 ± 0.5484 vs. 1.1419 ± 0.1104), p-eNOS-Thr496 (1.1013 ± 0.0767 vs.1.1049 ± 0.1115), VEGF (0.9856 ± 0.0913 vs. 0.9372 ± 0.1329), VEGFR2 (1.5369 ± 0.1827 vs. 1.4844 ± 0.2474), and p-VEGFR2 (0.4799 ± 0.0679 vs. 0.4146 ± 0.0252) protein expressions in BDL rats with 28-day vehicle (n = 8) or ascorbate (n = 7) treatment are shown in Fig. 3. There was no significant difference between the two groups (all $p > 0.05$).

3.2.3. Fibrosis area ratio

The fibrosis area ratio determined by Sirius red quantification was not significantly different between BDL rats with 28-day vehicle (n = 8) or ascorbate (n = 8) treatment (12.38 ± 0.77% vs. 11.60 ± 0.37%, $P > 0.05$). The representative hepatic Sirius red staining images are shown at the lower right panel of Fig. 3.

3.2.4. Hepatic hydroxyproline content

The hydroxyproline content was not significantly different between BDL rats with 28-day vehicle (n = 8) or ascorbate (n = 8) treatment (1.94 ± 0.16 vs. 1.51 ± 0.40 μg/mg of liver, $p > 0.05$).

3.3. Hepatic and collateral vasoresponsiveness study

3.3.1. Hepatic ET-1 vasoresponsiveness

(i) Direct incubation effect

There was no significant difference of baseline BW (vehicle (n = 6) vs. ascorbate (n = 7): 404.1 ± 6.5 vs. 380.8 ± 11.4 g), MAP (115.8 ± 2.5 vs. 116.1 ± 3.6 mmHg), HR (383 ± 16 vs. 376 ± 11 bpm) and PP (14.99 ± 0.92 vs. 15.23 ± 1.2 mmHg) between BDL rats preincubated with vehicle or ascorbate (all P > 0.05). Fig. 4(A) shows that there was no significant difference of perfusion pressure change between the two groups (all $p > 0.05$ for different concentrations of ET-1).

(ii) Acute treatment effect

There was no significant difference in baseline BW (vehicle (n = 5) vs. ascorbate (n = 5): 350.4 ± 13.2 vs. 386.6 ± 16.1 g), MAP (131.8 ± 7.3 vs. 121.18 ± 2.2 mmHg), HR (396 ± 9 vs. 378 ± 17 bpm) and PP (16.7 ± 0.9 vs. 18.0 ± 1.2 mmHg) between two groups, 60 min after vehicle or ascorbate injection (all $p > 0.05$). Fig. 4(B) reveals that there was no significant difference of perfusion pressure change between the two groups (all $p > 0.05$ for different concentrations of ET-1).

3.3.2. Portosystemic collateral vascular responsiveness to AVP in cirrhotic rats

(i) Direct incubation effect

There was no significant difference of BW (vehicle (n = 5) vs. ascorbate (n = 7): 376.2 ± 22.6 vs. 379.1 ± 28.6 g), MAP

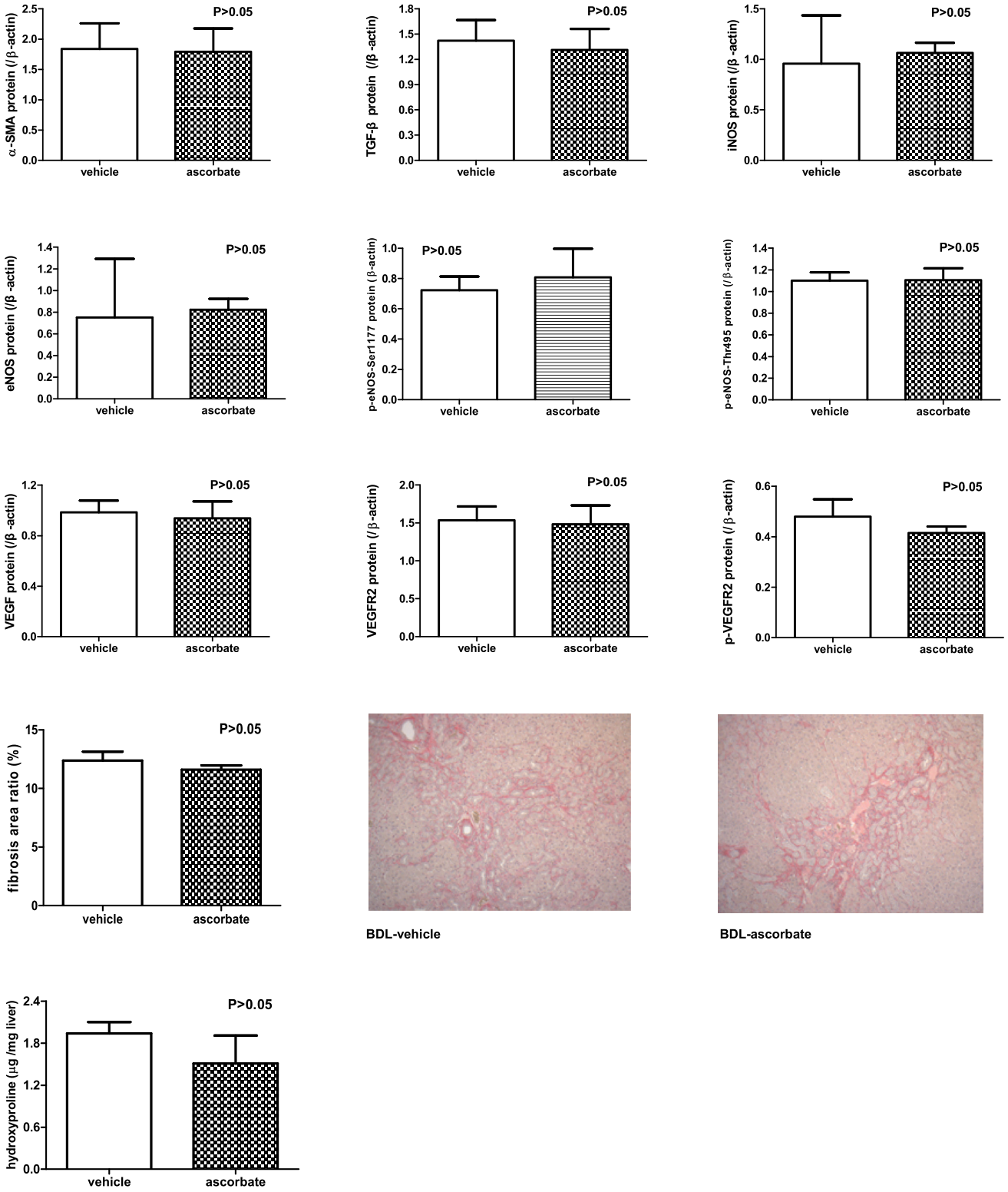

Fig. 3. Hepaticα-SMA, TGF-β, iNOS, eNOS, p-eNOS-Ser1177, p-eNOS-Thr496, VEGF, VEGFR2, and p-VEGFR2 protein expressions. There was no significant difference between BDL rats with 28-day vehicle or ascorbate treatment (all $p > 0.05$). The lower panel reveals that the fibrosis area ratio determined by Sirius red staining was not significantly different between BDL rats with 28-day vehicle or ascorbate treatment ($p > 0.05$). The representative images are shown right. The lowest panel shows that the hepatic hydroxyproline content was not significantly different, either ($p > 0.05$).

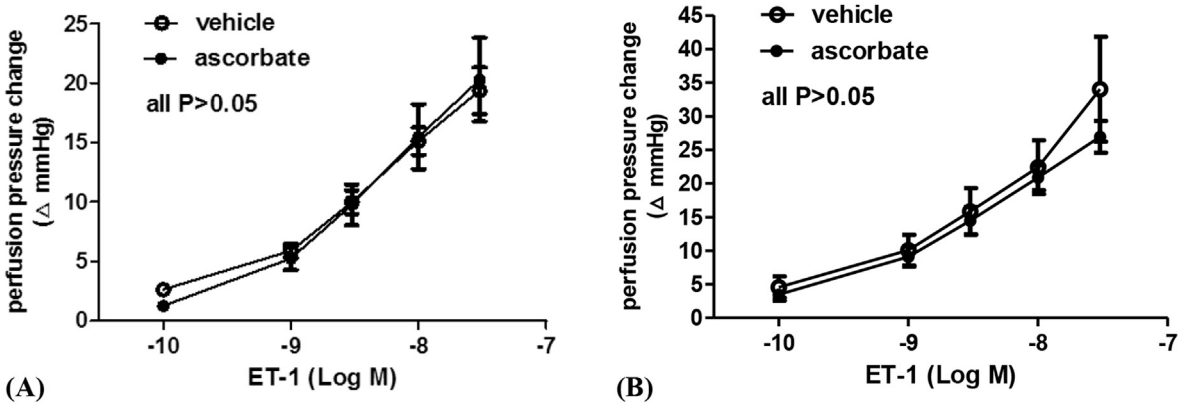

Fig. 4. Hepatic perfusion pressure changes of BDL rats with (A) vehicle or ascorbate preincubation; (B) vehicle or ascorbate injection. There was no significant difference for different concentrations of ET-1 (all $p > 0.05$).

(124.0 ± 4.4 vs. 125.1 ± 6.4 mmHg), HR (380.4 ± 16.1 vs. 350.1 ± 10.6 bpm) and PP (17.1 ± 0.7 vs. 18.4 ± 0.9 mmHg) between BDL rats with vehicle or ascorbate preincubation (all $p > 005$). Fig. 5(A) reveals that there was no significant difference of the perfusion pressure changes between the two groups (P > 0.05).

(ii) Acute treatment effect

There was no significant difference of BW (vehicle (n = 6) vs. ascorbate (n = 6): 371.5 ± 9.3 vs. 373.3 ± 2.3 g), MAP (108.1 ± 4.8 vs. 109.7 ± 3.3 mmHg), HR (339 ± 20 vs. 346 ± 18 bpm), PP (18.1 ± 1.1 vs. 17.2 ± 0.8 mmHg) (all $p > 0.05$), 60 min after vehicle or ascorbate injection. Fig. 5(B) shows that there was no significant difference in the perfusion pressure changes between the two groups ($p > 0.05$).

3.4. Shunting and mesenteric angiogenesis

3.4.1. Portosystemic shunting degree

The upper panel of Fig. 6 depicts that there was no significant difference of shunting degree between BDL rats with 28-day vehicle (n = 10) or ascorbate (n = 9) treatment (69.75 ± 2.50% vs. 56.14 ± 6.39%, $p > 0.05$).

3.4.2. Mesenteric vascular density

The upper panel of Fig. 6 shows that the mesenteric vascular length per window area (0.0123 ± 0.0024 vs. 0.0101 ± 0.0031/μm, $p > 0.05$) and vascular area per window area (5.7290 ± 1.4440 vs. 4.8380 ± 1.4840, $p > 0.05$) were not significantly different between BDL rats with 28-day vehicle (n = 8) or ascorbate (n = 8) treatment.

3.4.3. Mesenteric angiogenic factors protein expression

The lower panel of Fig. 6 depicts the mesenteric protein expressions of VEGF (/β-actin: 1.2918 ± 0.2051 vs. 1.7161 ± 0.2519), VEGFR2 (0.8083 ± 0.0631 vs. 0.7375 ± 0.1045), p-VEGFR2 (0.7958 ± 0.1215 vs.1.2086 ± 0.2056), PDGF (0.9558 ± 0.1723 vs. 1.3298 ± 0.2261), PDGFRβ (1.7727 ± 0.6128 vs. 2.9158 ± 0.9141), COX1 (1.1110 ± 0.0990 vs. 1.1372 ± 0.1451), COX2 (0.9088 ± 0.1476 vs. 0.7413 ± 0.1237), eNOS (2.6153 ± 0.7186 vs. 2.9311 ± 0.6676), p-eNOS-Thr495 (0.7231 ± 0.0905 vs. 0.8085 ± 0.1897), and p-eNOS-Ser1177 (0.7231 ± 0.0905 vs. 0.8085 ± 0.1897). They were not significantly different between the two groups (n = 8 for each group) (all $p > 0.05$).

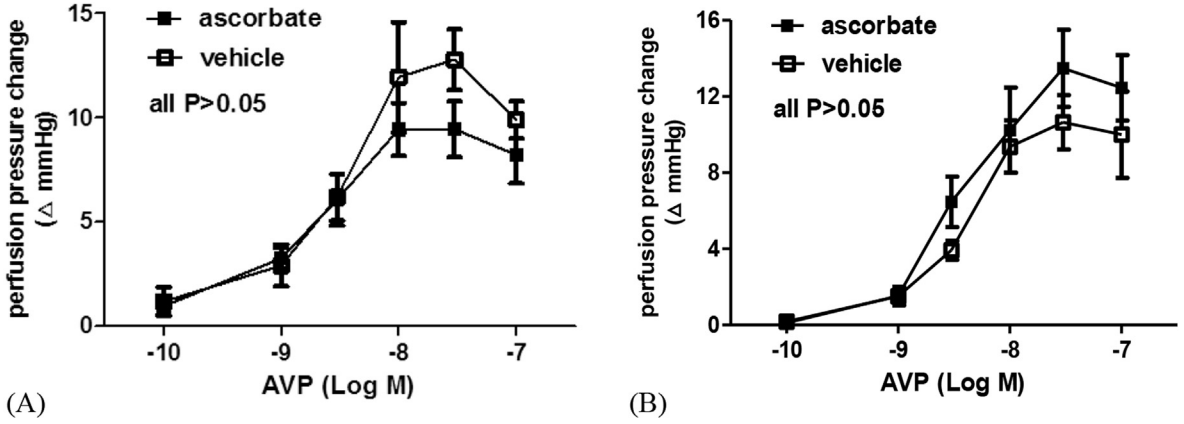

Fig. 5. Portosystemic collateral perfusion pressure changes of BDL rats with (A) vehicle or ascorbate preincubation; (B) Acute vehicle or ascorbate injection. There was no significant difference for different concentrations of AVP (all $p > 0.05$).

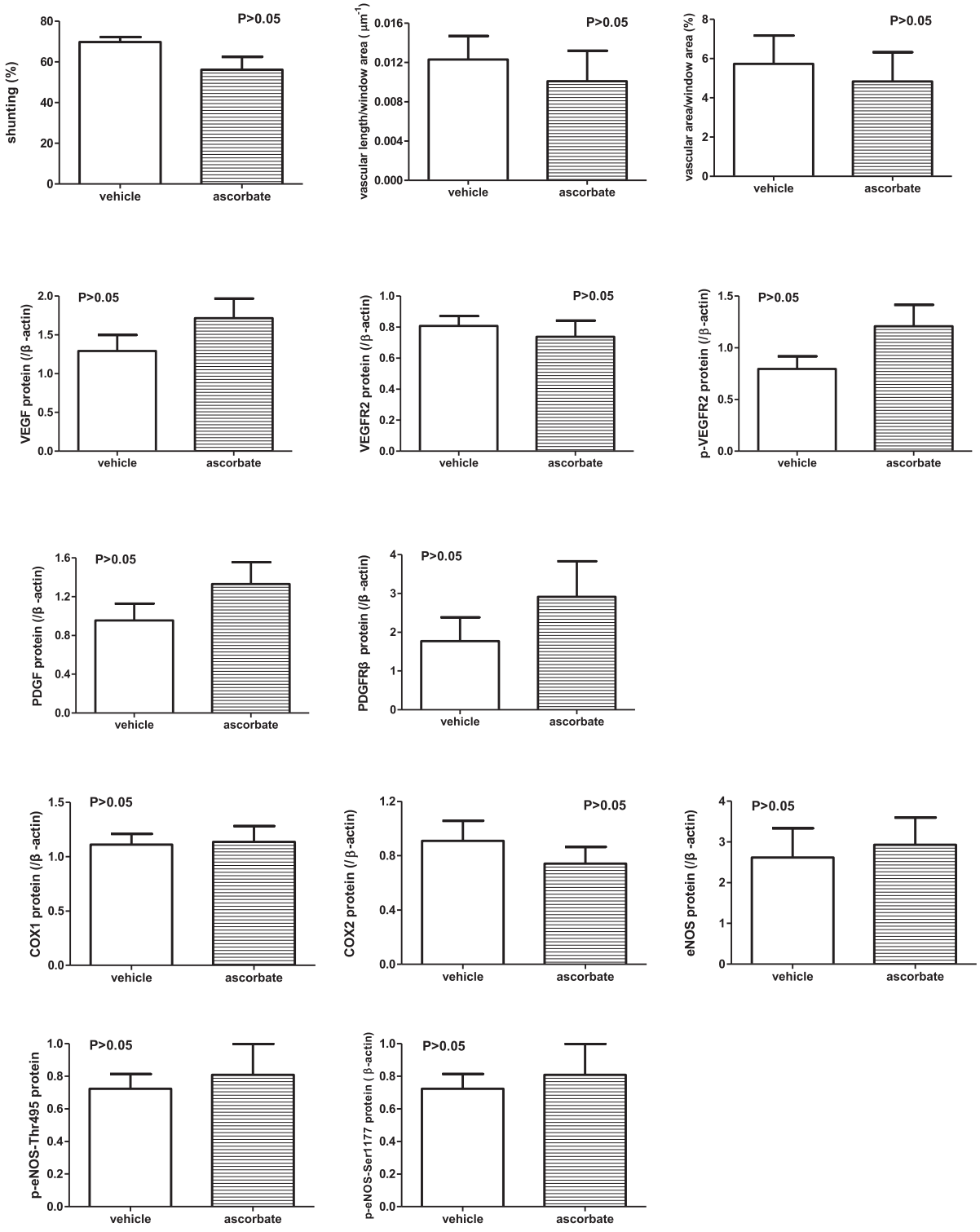

Fig. 6. Upper panel: Portosystemic shunting and mesenteric window vascular density (length and area) in BDL rats with vehicle or ascorbate treatment. There was no significant difference between the two groups ($p > 0.05$). Lower panel: The mesenteric VEGF, VEGFR2, p-VEGFR2, PDGF, PDGFβ, COX1, COX2, eNOS, p-eNOS, p-eNOS-Ser1177 protein expressions in BDLrats with vehicle or ascorbate treatment. There was no significant difference between the two groups (all $p > 0.05$).

4. Discussion

The excess accumulation of extracellular matrix (ECM) is an action attempting to compensate for liver parenchymal injury. Unfortunately, persistent fibrogenesis is a major driving force of liver cirrhosis.[47] Regarding the effects of ascorbate on hepatic fibrogenesis, Abhilash et al. found that the anti-fibrotic action of ascorbate in alcoholic liver fibrosis might be

attributed to the amelioration of oxidative stress. The mRNA expressions of hepatic fibrotic genes CYP2E1, TGF-β1, TNF-α and α1(I) collagen were down-regulated by ascorbate.[48] Furthermore, ascorbate decreased T-cell leukemia cells via TGF-α down-regulation.[49] Ascorbate also inhibited the gene expressions of bFGF, VEGF and MMP-2 in BALB/C mice implanted with sarcoma 180 cancer cells.[50] Conversely, ascorbate stimulated cell proliferation and inhibited MMP and TGF-β expression at higher concentrations.[50] Ascorbate also induced collagen production.[51] Furthermore, in guinea pigs, animals exhibiting the same genetic defect of ascorbate biosynthesis as humans, mild scorbutic conditions resulted in a decrease in TGF-β1 expression in blood plasma, implicating the diverse actions towards fibrogenesis.[52] The current study revealed that ascorbate did not influence ALT, AST, hepatic hydroxyproline content, liver fibrosis ratio, hepatic protein expressions of α-SMA, TGF-β, iNOS, eNOS, p-eNOS-Ser1177, p-eNOS-Thr496, VEGF, VEGFR2, p-VEGFR2, and liver histology. The different results may be ascribed to different experimental design, animal species, dose of ascorbate and diseases models. Another concern is that the cholestatic liver injury could be multi-factorial. Additionally, the hepatic oxidative stress parameters were not influenced by ascorbate. BDL has been recognized to induce significant oxidative stress. It is therefore inferred that the oxidative stress induced by this animal model might be too severe to be modulated by ascorbate.

The systemic and portal hemodynamic parameters were also not influenced by ascorbate. Regarding the systemic circulation, it is possible that ascorbate did not influence the cardiac performance and vascular resistance within this experimental setting. In addition, PP is collectively determined by liver resistance, splanchnic blood flow with SMA flow as the index, and portosystemic collateral vascular resistance. The lack of change in PP can be explained by the results that ascorbate did not modify liver fibrosis, SMA flow and collateral vasoresponsiveness.

In an animal study, ascorbate relaxed rat and guinea pig aortic rings that were pre-contracted with phenylephrine or potassium chloride.[53] However, in the present study, the intrahepatic ET-1 vasoresponsiveness and collateral AVP vasoresponsiveness was not influenced by ascorbate, either by direct incubation or *in vivo* injection. Although the different findings may be ascribed to different animal models, experimental settings, and dose of ascorbate, ascorbate may not be able to dilate the hepatic and collateral vascular beds, which has not been reported in the past.

On the molecular level, a previous study indicated that ascorbate dose-dependently increased eNOS-Ser1177 phosphorylation, and concomitantly decreased eNOS-Thr495 phosphorylation as early as 5 min after ascorbate administration; this was a phosphorylation pattern indicative of increased eNOS activity.[54] This effect was at least partly owing to activation of AMP-activated kinase, although another study claimed that ascorbate did not induce the expression of eNOS protein, nor did it induce de novo synthesis of NOS.[55] Moreover, it has been reported that ascorbate enhanced NO synthesis in lipopolysaccharide-and interferon-gamma-activated macrophage J774.1 cells through the iNOS pathway.[56] However, the current study reveals that hepatic iNOS, eNOS and eNOS phosphorylation were not modified by ascorbate. This provides the evidence, at least partially, for the lack of vascular effect of ascorbate on hepatic vascular bed. Taken together, the present data suggest that ascorbate lacks remarkable impacts on the intrahepatic vasculature, although further clarification is needed.

The previous study has identified the anti-angiogenesis effect of ascorbate. The level of the main pro-angiogenic factor VEGF was significantly higher in scorbutic mice challenged with melanoma B16FO cells than in ascorbate-supplemented mice.[27] A previous study indicated a significant inverse correlation between ascorbate and VEGF protein levels.[28] It has also been found that L:-ascorbate inhibited the secretion of vascular VEGF and the growth of xenograft pancreatic tumor in athymic mice.[29] However, the mesenteric angiogenesis and proangiogenic factors protein expressions were not significantly influenced by ascorbate. Furthermore, the portosystemic shunting degree was also not modulated. The results suggest that ascorbate does not exert a significant impact in pathological angiogenesis in cirrhosis with portal hypertension.

In conclusion, ascorbate, either acute or chronic treatment, does not influence hepatitis, fibrosis, systemic/portal hemodynamic parameters, hepatic/collateral vascular responsiveness, mesenteric angiogenesis and severity of shunting in rats with BDL-induced cholestatic liver injury. However, this model of hepatic damage could be multifactorial and excessively overwhelming to be alleviated by ascorbate.

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References

1. Iwakiri Y, Groszmann RJ. Vascular endothelial dysfunction in cirrhosis. *J Hepatol* 2007;**46**:927–34.
2. Iwakiri Y, Groszmann RJ. The hyperdynamic circulation of chronic liver diseases: from the patient to the molecule. *Hepatology* 2006;**43**: S121–31.
3. Hsu SJ, Lee FY, Wang SS, Hsin IF, Lin TY, Huang HC, et al. Caffeine ameliorates hemodynamic derangements and portosystemic collaterals in cirrhotic rats. *Hepatology* 2015;**61**:1672–84.
4. Fernandez M, Vizzutti F, Garcia-Pagan JC, Rodes J, Bosch J. Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice. *Gastroenterology* 2004;**126**: 886–94.
5. Mejias M, Garcia-Pras E, Tiani C, Miquel R, Bosch J, Fernandez M. Beneficial effects of sorafenib on splanchnic, intrahepatic, and

portocollateral circulations in portal hypertensive and cirrhotic rats. *Hepatology* 2009;**49**:1245–56.
6. Poli G, Cutrin JC, Biasi F. Lipid peroxidation in the reperfusion injury of the liver. *Free Radic Res* 1998;**28**:547–51.
7. Jayatilleke A, Shaw S. Stimulation of monocyte interleukin-8 by lipid peroxidation products: a mechanism for alcohol-induced liver injury. *Alcohol* 1998;**16**:119–23.
8. Poli G, Parola M. Oxidative damage and fibrogenesis. *Free Radic Biol Med* 1997;**22**:287–305.
9. Chojkier M, Houglum K, Lee KS, Buck M. Long- and short-term D-alpha-tocopherol supplementation inhibits liver collagen alpha1(I) gene expression. *Am J Physiol* 1998;**275**:G1480–5.
10. Esrefoglu M. Oxidative stress and benefits of antioxidant agents in acute and chronic hepatitis. *Hepatol Mon* 2012;**12**:160–7.
11. Niki E. Interaction of ascorbate and a-tocopherol. *Ann N Y Acad Sci* 1987; **498**:186–99.
12. Bendich A, D'Apolito P, Gabriel E, MachIm U. Interaction of dietary vitamin C and vitamin E on Guinea pig immune responses to mitogens. *J Nutr* 1984;**ll4**:1588–93.
13. Cash WJ, McCance DR, Young IS, McEneny J, Cadden IS, McDougall NI, et al. Primary biliary cirrhosis is associated with oxidative stress and endothelial dysfunction but not increased cardiovascular risk. *Hepatol Res* 2010;**40**:1098–106.
14. Yamamoto Y, Yamashita S, Fujisawa A, Kokura S, Yoshikawa T. Oxidative stress in patients with hepatitis, cirrhosis, and hepatoma evaluated by plasma antioxidants. *Biochem Biophys Res Commun* 1998;**247**: 166–70.
15. Simon JA. Vitamin C and cardiovascular disease: a review. *J Am Coil Nutr* 1992;**11**:107–25.
16. Heitzer T, Just H, Munzel T. Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. *Circulation* 1996;**94**:6–9.
17. Regensteinera JG, Popylisen S, Bauer TA, Lindenfeld JA, Gill E, Smith S, et al. Oral l-arginine and vitamins E and C improve endothelial function in women with type 2 diabetes. *Vasc Med* 2003;**8**:169–75.
18. Motoyama T, Kawano H, Kugiyama K, Hirashima O, Ohgushi M, Yoshimura M, et al. Endothelium-dependent vasodilation in the brachial artery is impaired in smokers: effect of vitamin C. *Am J Physiol* 1997;**273**: H1644–50.
19. Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, et al. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci U S A* 1996;**93**:3704–9.
20. Dhariwal KR, Hartzell WO, Levine M. Ascorbic acid and dehydroascorbic acid measurements in human plasma and serum. *Am J Clin Nutr* 1991;**54**:712–6.
21. Ting HH, Timimi FK, Boles KS, Creager SJ, Ganz P, Creager MA. Vitamin C improves endothelium dependent vasodilation in patients with non-insulin- dependent diabetes mellitus. *J Clin Invest* 1996;**97**:22–8.
22. Hofmann H, Schmidt HH. Thiol dependence of nitric oxide synthase. *Biochemistry* 1995;**34**:13443–52.
23. Hornig B, Arakawa N, Kohler C, Drexler H. Vitamin C improves endothelial function of conduit arteries in patients with chronic heart failure. *Circulation* 1998;**97**:363–8.
24. Mietus-Snyder M, Malloy MJ. Endothelial dysfunction occurs in children with two genetic hyperlipidemias: improvement with antioxidant vitamin therapy. *J Pediatr* 1998;**133**:35–40.
25. Gokce N, Keaney Jr JF, Frei B, Holbrook M, Olesiak M, Zachariah BJ, et al. Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation* 1999;**99**:3234–40.
26. Mikirova NA, Casciari JJ, Riordan MH. Ascorbate inhibition of angiogenesis in aortic rings ex vivo and subcutaneous Matrigel plugs in vivo. *J Angio Res* 2010;**2**:2.
27. Cha J, Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. Ascorbate supplementation inhibits growth and metastasis of B16FO melanoma and 4T1 breast cancer cells in vitamin C-deficient mice. *Int J Oncol* 2013;**42**:55–64.
28. Gokce N, Keaney Jr JF, Frei B, Holbrook M, Olesiak M, Zachariah BJ, et al. Low ascorbate levels are associated with increased hypoxia-inducible factor-1 activity and an aggressive tumor phenotype in endometrial cancer. *Cancer Res* 2010;**70**:5749–58.
29. Chen C, Sun J, Liu G, Chen J. Effect of small interference RNA targeting HIF-1alpha mediated by rAAV combined L:-ascorbate on pancreatic tumors in athymic mice. *Pathol Oncol Res* 2009;**15**:109–14.
30. Kountouras J, Billing BH, Scheuer PJ. Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. *Br J Exp Pathol* 1984;**65**: 305–11.
31. Cameron GR, Hasan SM. Disturbances of structure and function in the liver as the result of biliary obstruction. *J Pathol Bacteriol* 1958;**75**: 33–49.
32. Hsu SJ, Wang SS, Hsin IF, Lee FY, Huang HC, Huo TI, et al. Green tea polyphenol decreases the severity of portosystemic collaterals and mesenteric angiogenesis in rats with liver cirrhosis. *Clin Sci (Lond)* 2014; **126**:633–44.
33. Huang HC, Wang SS, Chen YC, Lee FY, Chang FY, Lin HC, et al. Cyclooxygenase expression in splanchnic hyposensitivity to glypressin of bleeding portal hypertensive rats. *Eur J Clin Invest* 2003;**33**:505–12.
34. Huang HC, Wang SS, Hsin IF, Chang CC, Lee FY, Lin HC, et al. Cannabinoid receptor 2 agonist ameliorates mesenteric angiogenesis and portosystemic collaterals in cirrhotic rats. *Hepatology* 2012;**56**:248–58.
35. Albillos A, Colombato LA, Groszmann RJ. Vasodilatation and sodium retention in prehepatic portal hypertension. *Gastroenterology* 1992;**102**: 931–5.
36. Hsu SJ, Hsin IF, Lin YL, Chen YC, Huang HC, Lee FY, et al. The influence of sorafenib on hepatic encephalopathy and the mechanistic survey in cirrhotic rats. *Eur J Clin Invest* 2012;**42**:1309–16.
37. Mittal MK, Gupta TK, Lee FY, Sieber CC, Groszmann RJ. Nitric oxide modulates hepatic vascular tone in normal rat liver. *Am J Physiol* 1994; **267**:G416–22.
38. Hamilton RL, Berry MN, Williams MC, Severinghaus EM. A simple and inexpensive membrane "lung" for small organ perfusion. *J Lipid Res* 1974;**15**:182–6.
39. Chan CC, Wang SS, Lee FY, Chang FY, Lin HC, Hou MC, et al. Effects of vasopressin on portal-systemic collaterals of cirrhotic rats. *Scand J Gastroenterol* 2005;**40**:83–9.
40. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;**72**:248–54.
41. Chojkier M, Groszmann RJ. Measurement of portal-systemic shunting in the rat by using γ-labeled microspheres. *Am J Physiol* 1981;**240**:G371–5.
42. Hodeige D, de Pauw M, Eechaute W, Weyne J, Heyndrickx GR. On the validity of blood flow measurement using colored microspheres. *Am J Physiol* 1999;**276**:H1150–8.
43. Muthuvel R, Venkataraman P, Krishnamoorthy G, Gunadharini DN, Kanagaraj P, Jone Stanley A, et al. Antioxidant effect of ascorbic acid on PCB (Aroclor 1254) induced oxidative stress in hypothalamus of albino rats. *Clin Chim Acta* 2006;**365**:297–303.
44. Fanucchi MV, Bracher A, Doran SF, Squadrito GL, Fernandez S, Postlethwait EM, et al. Post-exposure antioxidant treatment in rats decreases airway hyperplasia and hyperreactivity due to chlorine inhalation. *Am J Respir Cell Mol Biol* 2012;**46**:599–606.
45. Irwin DC, McCord JM, Nozik-Grayck E, Beckly G, Foreman B, Sullivan T, et al. A potential role for reactive oxygen species and the HIF-1alpha-VEGF pathway in hypoxia-induced pulmonary vascular leak. *Free Radic Biol Med* 2009;**47**:55–61.
46. Vissers MC, Gunningham SP, Morrison MJ, Dachs GU, Currie MJ. Modulation of hypoxia-inducible factor-1 alpha in cultured primary cells by intracellular ascorbate. *Free Radic Biol Med* 2007;**42**:765–72.
47. Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008;**134**:1655–69.
48. Abhilash PA, Harikrishnan R, Indira M. Ascorbic acid supplementation down-regulates the alcohol induced oxidative stress, hepatic stellate cell activation, cytotoxicity and mRNA levels of selected fibrotic genes in Guinea pigs. *Free Radic Res* 2012;**46**:204–13.

49. Harakeh S, Diab-Assaf M, Khalife JC, Abu-el-Ardat KA, Baydoun E, Niedzwiecki A, et al. Ascorbic acid induces apoptosis in adult T-cell leukemia. *Anticancer Res* 2007;**27**:289−98.
50. Yeom CH, Lee G, Park JH, Yu J, Park S, Yi SY, et al. High dose concentration administration of ascorbic acid inhibits tumor growth in BALB/C mice implanted with sarcoma 180 cancer cells via the restriction of angiogenesis. *J Transl Med* 2009;**7**:70.
51. Greesin J, Murad S, Pinnell S. Ascorbic acid stimulates collagen production without altering intracellular degradation in cultured human skin fibroblasts. *Biochem Biophys* 1985;**866**:272−4.
52. Ivanov VO, Roomi MW. Decreased expression of transforming growth factor β1 in blood plasma of Guinea pigs fed vitamin C deficient diet. *Clin Chim Acta* 1998;**269**:219−21.
53. Chang KC, Chong WS, Sohn DR, Kwon BH, Lee IJ, Kim CY, et al. Endothelial potentiation of relaxation response to ascorbic acid in rat and Guinea pig thoracic aorta. *Life Sci* 1993;**52**:PL37−42.
54. Ladurner A, Schmitt CA, Schachner D, Atanasov AG, Werner ER, Dirsch VM, et al. Ascorbate stimulates endothelial nitric oxide synthase enzyme activity by rapid modulation of its phosphorylation status. *Free Radic Biol Med* 2012;**52**:2082−90.
55. Heller R, Münscher-Paulig F, Gräbner R, Till U. L-Ascorbic acid potentiates nitric oxide synthesis in endothelial cells. *J Biol Chem* 1999;**274**:8254−60.
56. Mizutani A, Tsukagoshi N. Molecular role of ascorbate in enhancement of NO production in activated macrophage-like cell line, J774.1. *J Nutr Sci Vitaminol (Tokyo)* 1999;**45**:423−35.