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Original Article

Effects of entecavir, partial splenic artery embolization, and salvia miltiorrhiza on patients with hypersplenism due to hepatitis B virus-related cirrhosis

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Abstract: Objective: The aim of the current study was to examine the effects of partial splenic artery embolization (PSE), entecavir (ETV), and salvia miltiorrhiza (SM) on patients with hypersplenism due to hepatitis B virus-related cirrhosis. Methods: A total of 170 patients with hypersplenism due to hepatitis B virus-related cirrhosis were assigned randomly into 3 groups, including ETV, PSE+ETV, and PSE+ETV+SM groups. Sizes of the spleen, diameters of the portal veins and blood flow velocity of the portal veins, white blood cells (WBC), platelets (PLT), hyaluronic acid (HA), type III procollagen (PC III), type IV collagen (IV-C), laminin (LN), interleukin 2 (IL-2), interferon-γ (IFN-γ), IL-10, transforming growth factor-β1 (TGF-β1), tissue inhibitor of metalloproteinases-1 (TIMP-1), and metalloproteinase-1 (MMP-1) levels were measured. Results: Compared to the ETV group, WBC, PLT, IFN-γ, and MMP-1 significantly increased in the ETV+PSE group after treatment. They further significantly increased in the ETV+PSE+SM group, except WBC and PLT counts. Child-Pugh scores, diameters of the portal veins and blood flow velocity of the portal veins, HA, PC III, IV-C, LN, IL-10, TGF-β1, TIMP-1, and ratios of variceal hemorrhages 1 year post-treatment significantly decreased in the ETV+PSE group. They further significantly decreased in the PSE+ETV+SM group, except for diameter of the portal veins and blood flow velocity of the portal veins. There were no significant differences between ratios of complications in ETV+PSE and ETV+PSE+SM groups. Conclusion: Entecavir is effective for treatment of hypersplenism due to hepatitis B virus-related cirrhosis. PSE demonstrated improvements in therapeutic effects. Salvia miltiorrhiza can further improve therapeutic effects.

Keywords: Partial splenic artery embolization, entecavir, salvia miltiorrhiza, hypersplenism, hepatitic cirrhosis

Introduction

Chronic hepatitis B (CHB) virus (HBV) infection is a complex disease, with 15%-40% of infected persons progressing to severe liver diseases, including cirrhosis, liver failure, and hepatocellular carcinoma (HCC) [1]. Liver cirrhosis can often lead to the development of ascites and formation of varicose veins in the esophagus and proximal stomach. Long-term nucleos(t)ide analogue therapy for patients with CHB can prevent or delay development of long-term complications, including decompensated cirrhosis, CHB-related deaths, and CHB-related HCC [2]. Entecavir has been recommended as the first-choice drug for treatment of chronic hepatitis B, due to effectiveness and safety levels. Partial embolization of splenic vessels has been used for treatment of hypersplenism of thrombocytopenia in liver cirrhosis patients. Miyazaki et al. reported that PSE might be a safe and effective alternative to splenectomy procedures for treatment of thrombocytopenia [3, 4]. Salvia miltiorrhizae, a herb from the Traditional Chinese Medicine (TCM) pharmacopeia, can improve microcirculation, scavenge oxygen free radicals, regulate the metabolism of inflammatory lipid mediators, and decrease levels of inflammatory mediators [5]. Loads of evidence have proven that patients could obtain meaningful benefits, such as prolonging transplant-free survival or decreased complications, including variceal hemorrhages, by effectively treating hypersplenism [6]. The present study

Table 1. Background factors of patients

Group	N	Age	Gender (male/female)	Child-pugh classification (A/B)
ETV	58	41.6±11.2	38/20	30/28
PSE+ETV	68	40.8±12.6	32/36	37/31
PSE+ETV+SM	44	39.8±10.9	23/21	24/20
F/X^2		0.645	4.446	0.116
P		0.712	0.108	0.944

PSE: Partial splenic artery embolization; ETV: Entecavir; SM: Salvia miltiorrhiza.

aimed to evaluate the effects of partial splenic artery embolization, entecavir, and salvia miltiorrhiza on patients with hypersplenism due to hepatitis B virus-related cirrhosis.

Materials and methods

Subjects

Between January 2006 and June 2017, 170 patients (92 males, 78 females), aged 33-65 years (average 42.5±16.8 years), with hypersplenism due to hepatitis B virus-related cirrhosis were assigned randomly into 3 groups: (1) ETV (n=58); (2) PSE+ETV (n=68); and (3) PSE+ETV+SM (n=44). A total of 20 healthy volunteers were enrolled as the control group. The protocol was approved by the Institutional Ethics Committee of Southern Medical University. All patients provided written informed consent for enrollement. Demographic data of the patients are shown in **Table 1**. Demographic, data, age, ratio of gender and ratio of child-pugh classification were not statistically different between the 3 groups. Diagnosis of hypersplenism was established by a review of each patient's medical history, clinical laboratory data, ultrasonography results and CT examinations. Diagnosis of hepatic cirrhosis was confirmed by biopsies and pathological examinations. Eligibility criteria included: (1) Documented hypersplenism caused by cirrhosis, established by a review of the patient's history, CT results, or ultrasound; (2) Hepatitis B virus-related cirrhosis; (3) Leukopenia (white blood cells count $\leq 4.0\times10^9$/L), thrombocytopenia (platelet count $\leq 100\times10^9$/L), or both. Patients with severe jaundice (total serum bilirubin level $\geq$ 81.4 μmol/L) or spontaneous bacterial peritonitis were excluded.

Methods

ETV and basic treatment

ETV (Shanghai Squibb Pharmaceutical Company Limited, Shanghai, China) at 0.5 mg was orally administered once a day. Basic therapy included batilol tablets, at 50 mg, orally administered three times a day, vitamin B4 tablets, at 10 mg, three times a day, and propranolol, at 10 mg, twice a day.

Salvia miltiorrhiza treatment

This treatment involved administration of salvia miltiorrhiza tablets (Wuhan Hualong Pharmaceutical Company Limited, Wuhan, China.) at 200 mg twice a day.

Partial splenic artery embolizationy

Briefly, a 5-Fr diagnostic catheter (Terumo, Tokyo, Japan) was inserted into the femoral artery using the Seldinger method. After celiac angiography and selective splenic arterial angiography procedures were routinely performed to observe the distribution of splenic arteries and collateral circulation routes, the tip of the catheter was placed as distal as possible at the hilus of the spleen. Embolization was performed using embosphere microspheres (Biosphere Medical Inc, 1050 Hingham St, Rockland, USA). The extent of embolization was set at 50%~70% of the spleen. To achieve this, embolization was performed progressively by means of repeated injection embosphere microspheres under angiography control. Immediate angiography procedures were conducted after each injection. The extent of embolization was expressed as the percentage of the ablated splenic parenchyma area shown by post-embolization angiography (**Figure 1**).

Volumetry of the spleen [7]

All imaging examinations were performed with Multidetector Computed Tomography (MDCT) scanners (64-MDCT, Somatom Sensation, Siemens Healthcare) with 5-mm slice thickness and reconstruction interval. Images were acquired from the diaphragm to the pubic symphysis in the portal venous phase (70 seconds after initiation of contrast injection). A total of 125 mL of contrast material (iohexol-350, Omnipaque, GE Healthcare) was administered at

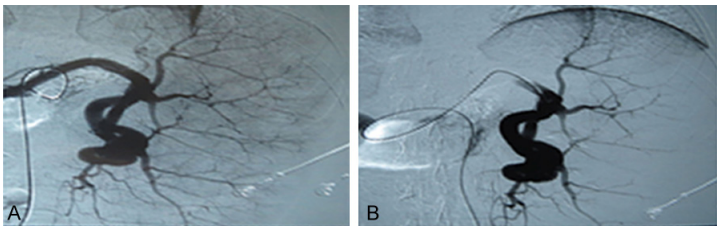

Figure 1. Image of splenic artery embolization. A. Before embolization; B. After embolization.

a rate of 3-4 mL/s, for a total iodine dose of 43.75 g. The contrast agent was administered through an antecubital vein with an 18- to 20-gauge catheter and a mechanical injector (Stellant, Medrad). According to the results of MDCT, values for splenic volume and longest one-dimensional diameters were automatically calculated with the software after segmentation of the spleen. Segmented splenic volume was quantified with the software as a count of the voxels within the segmented region. It was the reference standard for quantification of splenic volume in this study. Largest transaxial diameters were quantified to comply with the requirements of the formula for a prolate ellipsoid. Splenic width (W) was defined as the longest diameter of the spleen on transaxial slices and splenic thickness (T) was defined as the longest perpendicular diameter on the same transaxial slice that did not traverse the splenic parenchymal boundaries. Splenic length (L) was quantified as the vertical distance between the most superior and most inferior margins of the spleen. Splenic volume was calculated with the prolate ellipsoid formula by incorporating the product of one-dimensional diameters (W×T×L) into the equation: VE = (W×T×L×π/6).

Hemodynamic analysis

Ultrasonographic examinations were performed using HDI5000 and HPsono4500 color Doppler units with a 3.75-MHz convex probe. All patients and controls were fasted overnight prior to the procedure. They were examined in the supine position during quiet respiration. Measurements of diameter, flow direction, and flow velocity in the portal veins, splenic veins, and splenic arteries were done in all patients and controls. Blood flow was measured at the crossing point with the hepatic artery or just distally to it. Diameters of the blood vessels were calculated from the inner surface within the vessel, as seen in a longitudinal view. The sample volume was selected from 2 to 5 mm widths to include the width of the vessel. Flow direction was assessed according to the upward or downward position of the Doppler waveform over the baseline. Flow velocity was calculated as an average value of three cosecutive measurements.

Blood cells detection

The complete blood count was determined using Beckman Coulter LH750 Hematology Analyzer (C. E. Beckman Company, USA).

Identification of indexes of hepatic fibrosis

Indexes of hepatic fibrosis, hyaluronic acid, type III procollagen, type IV, and laminin were detected by radioimmunoassay (RIA), with RIA kits provided by Corgenix Inc. (Colorado, USA).

Identification of levels of hepatic fibrosis-relating cytokines

Peripheral blood samples of the patients were centrifuged to collect serum. The serum was dispensed in 50 μl aliquots and stored at -20°C. Levels of hepatic fibrosis-relating cytokines, such as IL-2, IFN-γ, IL-10, and TGF-β1, were determined using ELISA kits, according to manufacturer instructions (R&D).

Identification of concentrations of indexes of matrix degradation

Indexes of matrix degradation, TIMP-1 and MMP-1, were assayed using the two-site ELISA sandwich technique (Amersham Pharmacia Biotech, Little Chalfont, Buckinghampshire, England), with specific antibodies as a solid phase. MMP-1 assays recognized total human MMP-1, including free and combined with TIMP-1. TIMP-1 assays recognized total human TIMP-1, including free and combined with any metalloproteinases bound to the solid phase. TIMP-1 or MMP-1 bound to the solid phase was detected by peroxidase labelled antibodies. There was no cross-reactivity between TIMP-1 and MMP-1 in these assays.

Statistical analysis

Data were recorded using Statistical Product and Service Solutions (SPSS) 20.0 software

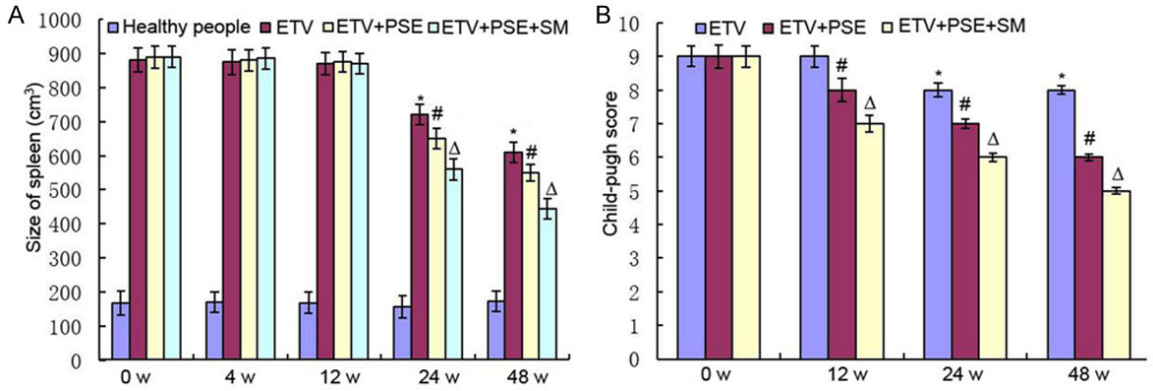

Figure 2. Sizes of spleens and changes of Child-Pugh scores. A. Sizes of spleen; B. Changes of Child-Pugh scores. * vs Healthy people group, P < 0.05; # vs ETV group, P < 0.05; Δ vs PSE+ETV group, P < 0.05. PSE: Partial splenic artery embolization; ETV: Entecavir; SM: Salvia miltiorrhiza.

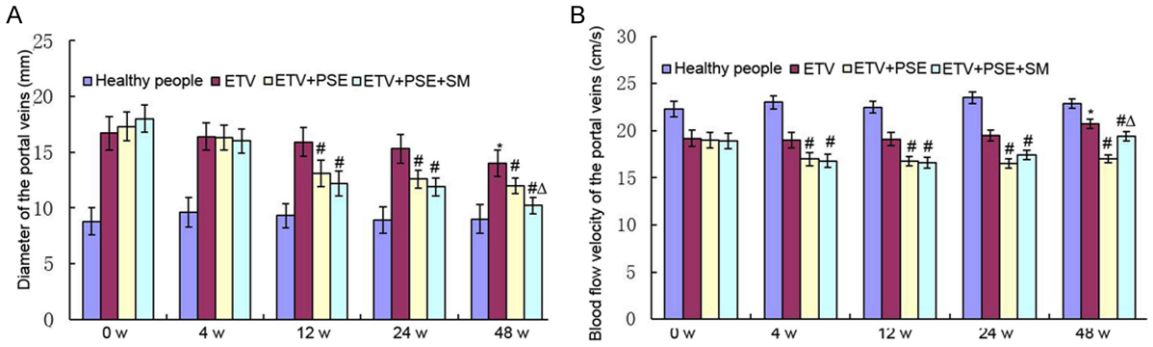

Figure 3. Changes in diameters of the portal veins and blood flow velocity of the portal veins. A. Diameter of the portal veins; B. Blood flow velocity the portal veins. * vs Healthy people group, P < 0.05; # vs ETV group, P < 0.05. PSE: Partial splenic artery embolization; ETV: Entecavir; SM: Salvia miltiorrhiza.

(IBM Corp., Armonk, NY, USA). Results are reported as mean ± standard deviation if normal distribution was met. Indicators at different points in one group were compared to determine correlation levels between repeated measurement data. Repeated measurement data were not correlated with a *P*-value > 0.05 by Mauchly's Test of Sphericity. These data met the requirement of Huynh-Feldt and were analyzed with one-way analysis of variance (ANOVA). Repeated measurement data were correlated if *P* values < 0.05. They were analyzed using ANOVA. Enumeration data were analyzed using Chi-square tests. *P* values < 0.05 indicate statistical significance.

Results

Sizes of spleens and changes in Child-Pugh scores

As shown in **Figure 2A**, compared to healthy people, sizes of spleens of the patients increased. Compared to the ETV group, sizes of spleens decreased significantly in the PSE+ETV group. They further significantly decreased in the PSE+ETV+SM group after treatment from 8 weeks until 48 weeks. As shown in **Figure 2B**, compared to the ETV group, Child-Pugh scores decreased significantly in the PSE+ETV group. They further significantly decreased in the PSE+ETV+SM group after treatment from 8 weeks until 48 weeks.

Diameters of the portal veins and blood flow velocity of the portal veins

As shown in **Figure 3A**, compared to healthy people, diameters of the portal veins of the patients increased. Compared to the ETV group, diameters of the portal veins decreased significantly in the PSE+ETV group and the PSE+ETV+SM group after treatment for 12 weeks. As shown in **Figure 3B**, compared to healthy people, blood flow velocity of the portals of the patients increased. Compared to

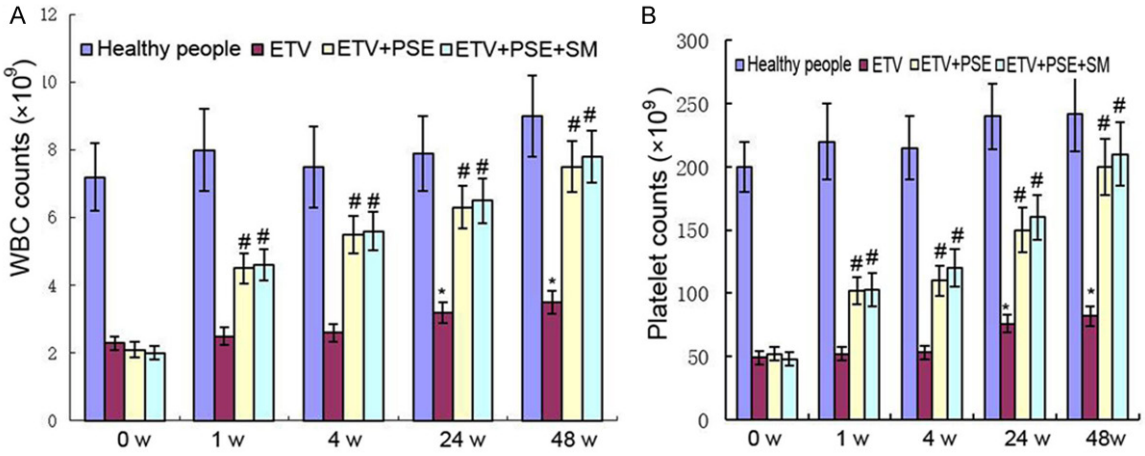

Figure 4. Changes in WBC and PLT counts. A. WBC counts; B. PLT counts. * vs Healthy people group, $P < 0.05$; # vs ETV group, $P < 0.05$; Δ vs PSE+ETV group, $P < 0.05$. PSE: Partial splenic artery embolization; ETV: Entecavir; SM: Salvia miltiorrhiza; WBC: White blood cell; PLT: Platelet.

the ETV group, blood flow velocity of the portal veins decreased significantly in the PSE+ETV group and PSE+ETV+SM group after treatment for 12 weeks.

Changes in WBC and PLT counts

As shown in **Figure 4A**, compared to healthy people, WBC counts of the patients decreased. Compared to the ETV group, WBC counts increased significantly in the PSE+ETV group and PSE+ETV+SM group after treatment from 1 week until 48 weeks. As shown in **Figure 4B**, compared to healthy people, PLT counts of the patients decreased. Compared to the ETV group, PLT counts increased significantly in the PSE+ETV group and PSE+ETV+SM group after treatment from 1 week until 48 weeks. However, there were no significant differences between the PSE+ETV group and PSE+ETV+SM group.

Changes in levels of hepatic fibrosis-relating cytokines

As shown in **Figure 5A**, compared to the ETV group, IL-2 levels decreased significantly in the PSE+ETV group. They further decreased in the PSE+ETV+SM group after treatment from 12 weeks until 48 weeks. As shown in **Figure 5B**, compared to the ETV group, IFN-γ levels increased significantly in the PSE+ETV group. They further significantly increased in the PSE+ETV+SM group after treatment from 12 weeks until 48 weeks. As shown in **Figure 5C**, compared to the ETV group, IL-10 levels decreased significantly in the PSE+ETV group. They further significantly decreased in the PSE+ETV+SM group after treatment from 12 weeks until 48 weeks. As shown in **Figure 5D**, compared to the ETV group, TGF-β1 levels decreased significantly in the PSE+ETV group. They further significantly decreased in the PSE+ETV+SM group after treatment from 12 weeks until 48 weeks.

Changes in indexes of hepatic fibrosis (HA, PC III, IV-C, and LN)

As shown in **Figure 6A**, compared to the ETV group, HA levels decreased significantly in the PSE+ETV group. They further significantly decreased in the PSE+ETV+SM group after treatment from 24 weeks until 48 weeks. As shown in **Figure 6B**, compared to the ETV group, PC III levels decreased significantly in the PSE+ETV group. They further significantly decreased in the PSE+ETV+SM group after treatment from 24 weeks until 48 weeks. As shown in **Figure 6C**, compared to the ETV group, IV-C levels decreased significantly in the PSE+ETV group. They further significantly decreased in the PSE+ETV+SM group after treatment from 24 weeks until 48 weeks. As shown in **Figure 6D**, compared to the ETV group, LN levels decreased decreased significantly in the PSE+ETV group. They further significantly decreased in the PSE+ETV+SM group after treatment from 24 weeks until 48 weeks.

Changes in concentrations of indexes of matrix degradation

As shown in **Figure 7A**, compared to the ETV group, TIMP-1 levels decreased significantly in

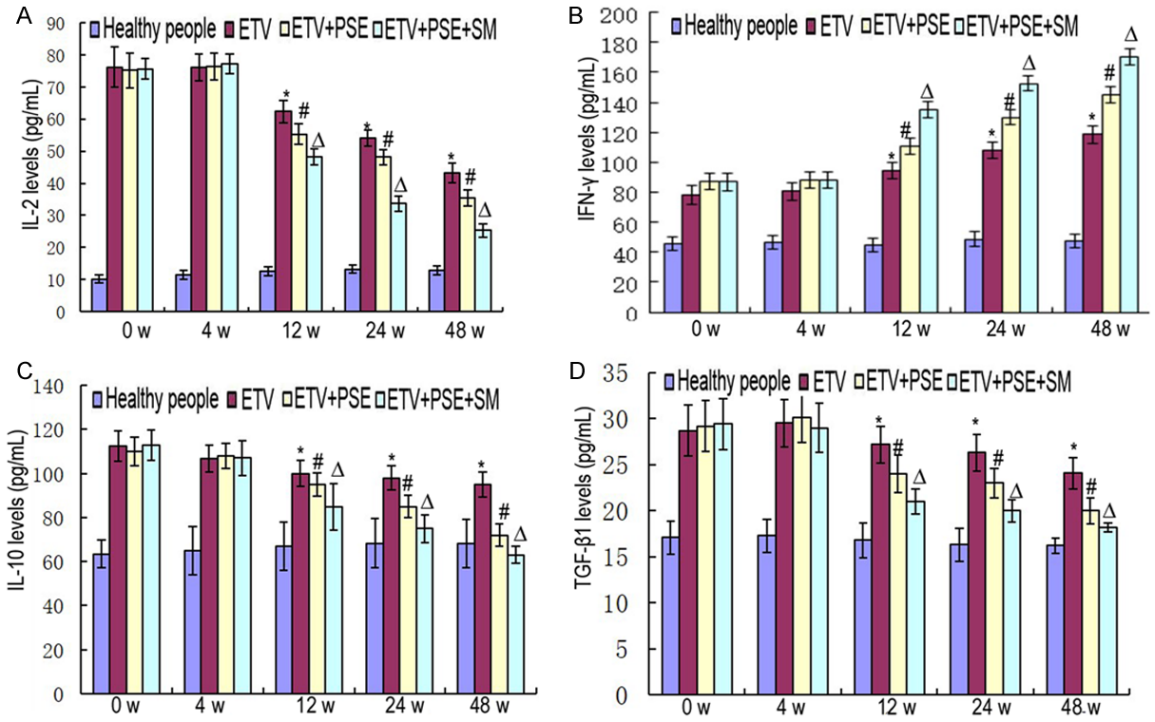

Figure 5. Changes in levels of hepatic fibrosis-relating cytokines. A. IL-2 levels; B. IFN-γ levels; C. IL-10 levels; D. TGF-β1 levels. * vs Healthy people group, $P < 0.05$; # vs ETV group, $P < 0.05$; Δ vs PSE+ETV group, $P < 0.05$. PSE: Partial splenic artery embolization; ETV: Entecavir; SM: Salvia miltiorrhiza; IL-2: Interleukin 2; IFN-γ: Interferon-γ; Interleukin 10: IL-10; TGF-β1: Transforming growth factor-β1.

the PSE+ETV group. They further significantly decreased in the PSE+ETV+SM group after treatment from 8 weeks until 48 weeks. As shown in **Figure 7B**, compared to the ETV group, MMP-1 levels increased significantly in the PSE+ETV group. They further significantly increased in the PSE+ETV+SM group after treatment from 8 weeks until 48 weeks.

Ratios of complications and post-treatment variceal hemorrhage

As shown in **Table 2**, there were no severe drug side effects in the 3 groups. Compared to the ETV group, ratios of complications increased significantly in the PSE+ETV group and PSE+ETV+SM group, but no differences were seen between the PSE+ETV group and PSE+ETV+SM group. Ratios of variceal hemorrhage decreased significantly in the PSE+ETV group and PSE+ETV+SM group. Patients recovered after they accepted the corresponding treatment for complications.

Discussion

HBV, a virus of the family hepadnaviridae, can be integrated into the host genome. It may lead to changes in genomic function or chromosomal instability. HBV hepatitis, characterized by diffused inflammatory reaction, is associated with cell damage and death. Mechanisms of cell damage are generally defined as the result of cytotoxic T-lymphocyte (CTL)-mediated immune response to viral infections [8]. In the development of hepatic cirrhosis, CD4 T-lymphocyte cells can regulate anti-tumor immunity by secreting lymphocyte cytokines, while CD8 T-lymphocyte cells can produce immunosuppressive cytokines to mediate immunosuppression. Th lymphocyte cells can produce two different kinds of cytokines. IFN-γ and IL-2 produced by Th1 cells can promote T-cell-mediated immune responses, playing an important role in antitumor immunity. IL-4 and IL-10, produced by Th2 cells, can inhibit Th1 cytokine release and promote immunoglobulin production.

A prolonged immune reactive phase, with multiple sequential flares of hepatitis or unremitting necro-inflammation, will result in progressive liver fibrosis, ultimately lead to cirrhosis. The pathogenesis of hepatic fibrosis involves significant deposition of fibrilae collagen and

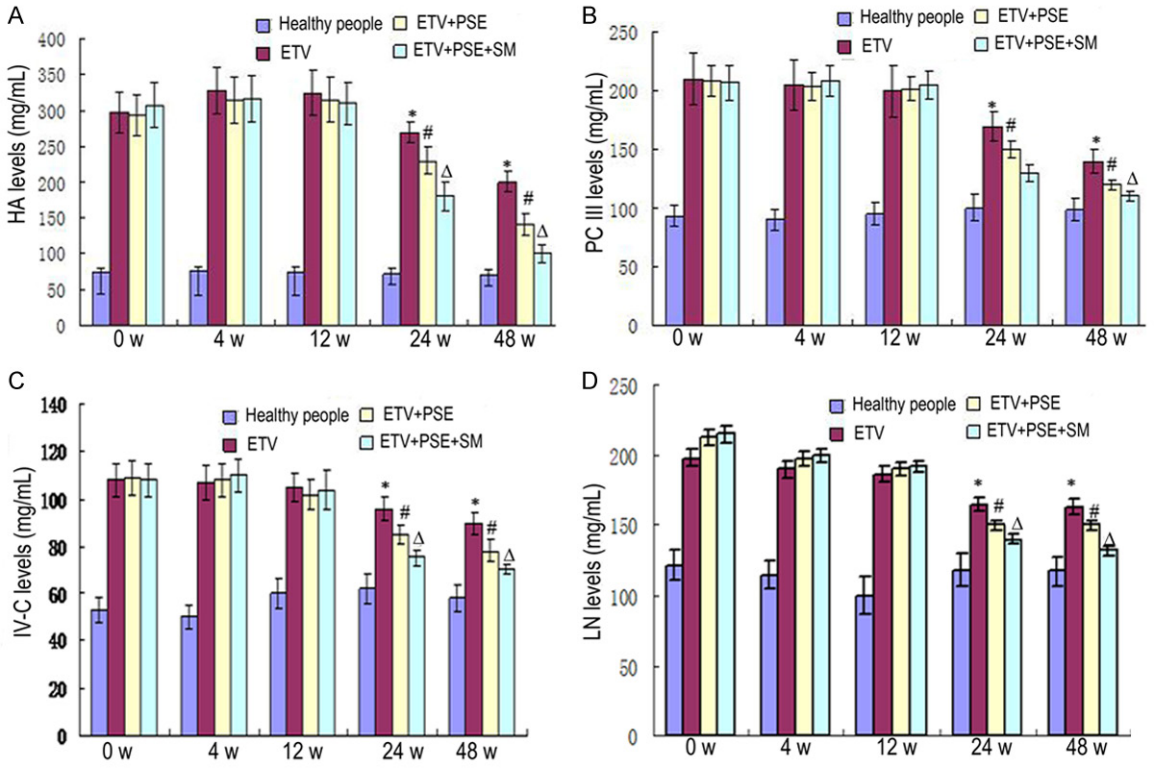

Figure 6. Changes in indexes of hepatic fibrosis. A. HA levels; B. PC III levels; C. IV-C levels; D. LN levels. * vs Healthy people group, P < 0.05; # vs ETV group, P < 0.05; Δ vs PSE+ETV group, P < 0.05. PSE: Partial splenic artery embolization; ETV: Entecavir; SM: Salvia miltiorrhiza; HA: Hyaluronic acid; PC III: Type III procollagen; IV-C: type IV collagen; LN: Laminin.

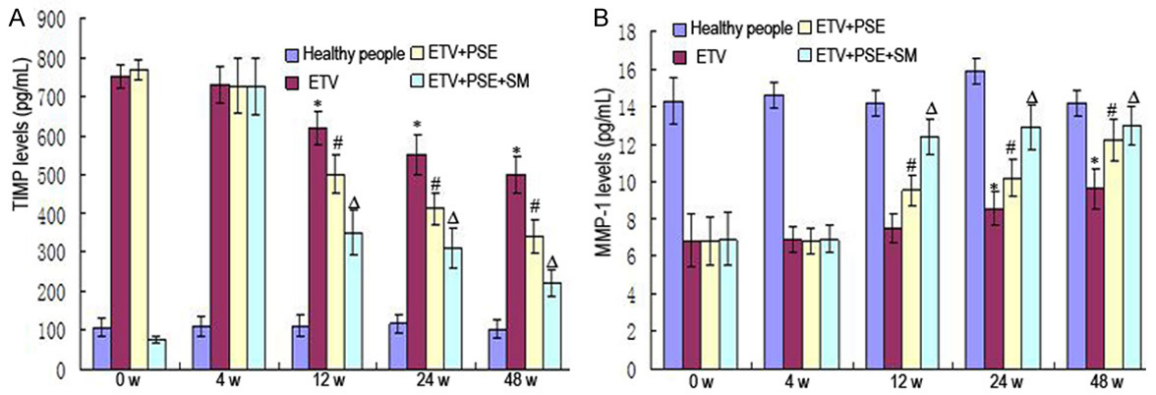

Figure 7. Changes in concentrations of indexes of matrix degradation. A. TIMP-1 levels; B. MMP-1 levels. * vs Healthy people group, P < 0.05; # vs ETV group, P < 0.05; Δ vs PSE+ETV group, P < 0.05. PSE: Partial splenic artery embolization; ETV: Entecavir; SM: Salvia miltiorrhiza; TIMP-1: Tissue inhibitor of metalloproteinases-1; MMP-1: Metalloproteinase-1.

other extracellular matrix proteins [9]. Liver fibrosis can distort the hepatic architecture, decrease the number of endothelial cells, and cause portal hypertension. Development of portal hypertension can be influenced by changes in resistance and blood flow in the hepatic vasculature. Increased resistance of portal blood flow in cirrhotic livers induces portal venous dilatation and congestion of portal venous flow, leading to elevated portal pressure. Key events are the activation and transformation of quiescent hepatic stellate cells into myofibroblast-like cells with the subsequent upregulation of proteins, such as α-smooth muscle actin, in-

Table 2. Rates of complications and post-treatment variceal hemorrhage [n (%)]

Group	n	Post-embolization syndrome	Splenic abscess	Pleural effusion	Ascites	Pneumonia	Variceal hemorrhage
ETV	58	-	-	-	-	-	6 (10.4)*
ETV+PSE	68	57 (83.8)*,#	2 (2.9)*,#	4 (5.9)*,#	3 (4.4)*	2 (2.9)*	4 (5.9)*,#
ETV+PSE+SM	44	35 (79.5)*,#	1 (2.3)*,#	2 (4.5)*,#	2 (4.5)*	2 (4.5)*	2 (4.5)*,#

* vs ETV group; # vs ETV+PSE group. PSE: Partial splenic artery embolization; ETV: Entecavir; SM: Salvia miltiorrhiza.

terstitial collagens, matrix metalloproteinases, and TIMP and proteoglycans [10]. Oxidative stress, a major contributing factor to the onset of liver fibrosis, is typically associated with a decrease in antioxidant defense. Mediators, such as TGF-β and platelet-derived growth factor (PDGF), are released from hepatocytes activated by lipid peroxidation products, intermediate metabolites of drugs or hepatotoxins, reactive oxygen species (ROS), cytokines and so forth [11].

Alterations in normal cell-cell and cell-matrix interactions play a significant role in the pathogenesis of hepatic fibrosis [12]. In fibrotic livers, significant quantitative and qualitative changes occur in the composition of epithelial-to-mesenchymal transition (ECM) in the periportal and perisinusoidal areas. Scars are typically composed of fibrillar collagen type I and III, proteoglycans, fibronectin and hyaluronic acid [13]. Chronic HBV infections can induce cell damage through an increased generation of ROS [15]. Oxidative stress, which favors mitochondrial permeability transition, can promote hepatocyte necrosis and/or apoptosis. The ROS generated can directly affect the HSC and myofibroblasts behavior [16]. ROS can upregulate expression of critical fibrosis-associated genes, such as COL1A1, COL1A2, MCP1 and TIMP-1 by activating signal transduction pathways and transcription factors including JNK, activator protein-1 and NF-κB [17]. ROS generation in HSC and myofibroblasts occurs in response to several pro-fibrogenic mediators, including angiotensin II, PDGF, TGF-β and leptin [18].

Chronic liver fibrosis can diminish ECM degradation, mainly through TIMP induction and MMP inhibition [19]. MMPs are the main enzymes responsible for ECM degradation, while TIMPs have the ability to inhibit MMPs. Activated Hepatic stellate cells (HSC) can not only synthesize and secrete ECM proteins, such as collagens type I and type III, but also produce MMP-1 and MMP-13, [20]. Activated HSC can upregulate expression and synthesis levels of TIMP-1 and TIMP2 [21]. TIMP-1 can not only prevent the degradation of the rapidly increasing ECM by blocking MMPs, but also inhibit the apoptosis of activated HSC [20]. Increased liver MMPs may lead to regression of fibrosis [22].

Entecavir (ETV), a deoxyguanosine analogue, is a highly effective and selective inhibitor of HBV replication. It can specifically inhibit hepadnaviral DNA polymerase by competing with the corresponding dNTP for incorporation in ascent DNA. ETV can also downregulate TGF-β1/TIMP-1 and upregulate MMP-1 activity [23]. PSE can not only relieve the symptoms of hypersplenism, but also reserve the spleen for immune function maintenance [24]. Salvia miltiorrhizae has been shown to reduce inflammation and immune response, as well as reduce the activation of ECM-producing cells and inhibit production of TGF-β1 [25, 26].

The current study found that ETV was effective for patients with hypersplenism due to hepatitis B virus-related cirrhosis. Compared to the ETV group, WBC, PLT, IFN-γ and MMP-1 significantly increased, while Child-Pugh scores, HA, PC III, IV-C, LN, IL-2, IL-10, TGF-β1 and TIMP-1 significantly decreased in the PSE+ETV group. Results demonstrated that PSE can improve therapeutic effects. Compared to the PSE+ETV group, IFN-γ and MMP-1 further significantly increased, while Child-Pugh scores, hyaluronic acid, type III procollagen, type IV collagen, laminin, IL-2, IL-10, TGF-β1, and TIMP-1 further significantly decreased in the PSE+ETV+SM. Results demonstrated that SM can further improve therapeutic effects. There were no drug side effects in the 3 groups. There were no significant differences in ratios of complications of the ETV+PSE group and ETV+PSE+SM group. The patients recovered after they acc-

epted the corresponding treatment for complications, which demonstrated that ETV and SM are safe drugs and that PSE is a safe therapeutic measure. In conclusion, entecavir is effective on treatment of hypersplenism due to hepatitis B virus-related cirrhosis. Moreover, PSE demonstrates improvements in therapeutic effects and SM can further improve therapeutic effects.

Disclosure of conflict of interest

None.

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