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Hepatoprotective Effects of Traditional Chinese Medicine on Liver Fibrosis from Ethanol Administration following Partial Hepatectomy

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Abstract

The aim of this study was to establish the effective hepatoprotective properties of traditional Chinese medicines (TCMs) in fibrotic rat liver regeneration after partial hepatectomy (PHx). Fibrosis was induced in rats by ethanol (EtOH, 5 ml/kg) administration for 6, 24, 72, and 168 h. The rats were then fed four TCMs (1 g/kg/day, *Codonopsis pilosula* (CP), *Salvia miltorrhiza Bunge* (SMB), *Bupleurum kasi* (BK), and *Elephantopus scaber L* (ESL)) to Spraque-Dawley rats for 6, 24, 72, and 168 h, respectively. Surgical 70% cirrhotic fibrosis PHx was then conducted at 6, 24, 72, and 168 h. The effects on liver regeneration were examined to estimate and measure hepatocyte growth factor (HGF), focal adhesion kinase (FAK), Cyclin D1, Cyclin E, and retinoblastoma protein (pRb) protein expression using Western blotting analysis. Cyclin D1, matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitors of metalloproteinase (TIMP)-1, TIMP-2 and TIMP-3 mRNA by Reverse transcription polymerase chain reaction (RT-PCR) were analyzed in cirrhotic fibrosis rats. transforming growth factor-β1 (TGF-β1), Cyclin D1, Cyclin E, pRb and E2F mRNA expression levels were determined in fibrotic rats following PHx using RT-PCR. We found elevated glutamyl oxaloacetic transaminase (GOT), glutamyl pyrubic transaminase (GPT), alkaline phosphatase (ALP), gammaglutamyl transpeptidase (γ-GT), glutathione (GSH) and nonprotein sulfhydryl (NPSH) and total bilirubin in serum after 6 h EtOH administration. These levels were progressively decreased over 168 h. Total protein and albumin were reduced in serum

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after 6 h administration and then progressively increased. In contrast, tissues disorder histology and morphology were determined in liver sections. After rats were fed TCMs we found that SMB extraction not only induced HGF, FAK, Cyclin D1, and pRb protein expression and Cyclin D1 mRNA increases, but also reduced MMP-2 and MMP-9 after 24 and 72 h post injury. In the cell cycle S phase the Cyclin E protein expression was increased by ESL. CP induced TIMP-1, TIMP-2 and TIMP-3 mRNA increases in fibrotic rats. We detected liver regeneration in fibrotic rats. We also found that the liver regeneration index increased from 6 to 168 h post PHx. After 168 h fibrotic liver regeneration rats exhibited reduced TGF-β1 mRNA expression and enhanced Cyclin D1, Cyclin E, pRb and E2F mRNA expression. TCMs play a crucial role in the early mediating process in fibrotic rat liver regeneration after PHx.

Key Words: fibrotic, hepatocyte growth factor, liver regeneration, partial hepatectomy, traditional Chinese medicine

Introduction

Traditional Chinese medicines (TCMs) are currently the world's most effective treatment for liver disease. They have shown positive effects in treating nearly every known form of liver disease including fibrosis, cirrhosis, hepatitis, and necroses. That is why they are used today. Almost all liver damage occurs due to drug and alcohol abuse. Some reports have indicated that TCMs also help prevent liver disease from occurring (1, 2). This study examined whether TCMs can protect the liver during major hepatectomy and ensure that the partial liver remnant is able to maintain sufficient function or not. We think TCMs could be applied as an anti-carcinogen and as a supportive treatment for liver damage from liver resection (3). When the liver undergoes surgical resection or suffers toxic injury, hepatocytes will start to regenerate using a compensatory growth process and then return to the non-proliferative state (4, 5, 9). However, much of the research into hepatic growth mechanisms has been performed using partial hepatectomy (PHx) animal models (17). Most liver cancer patients require partial liver section through surgery. After surgery the liver needs to grow to maintain liver mass (6). If the native hepatocyte proliferation function cannot be maintained the whole liver function integration after PHx will fail. Much of hepatic liver regeneration growth drug studies have been performed using PHx (7). This study focused on TCMs in facilitating cell protection progress in fibrosis liver regeneration.

Salvia miltorrhiza Bunge (SMB) is a widely used traditional Chinese herb in China. Some reports demonstrated that SMB promotes blood flow, removes blood stasis, stimulates cell survival signals, improves cell cycle control (1), inhibits cytokine inflammation (8) and improves anti-scar formation (11). It has been long used for treating liver disease in China. *Codonopsis pilosula* (CP) and *Bupleurum kasi* (BK) are important traditional Chinese crude drugs for treating hepatitis malaria and intermittent fever to resist cytokine and anti-fibrosis levels (19). *Elephantopus scaber L.* (ESL) is a folk medicine from Taiwan derived from the entire ESL E mollis H.B.K. and Pseudoelephantopus spicatos (Jass) plants. Rohr has hepatoprotective effects (29), anticancer effects on various cancer cells and induces cancer cell apoptosis from cell cycle arrest (23).

Growth factors such as hepatocyte growth factor (HGF) and transforming growth factor-β1 (TGF-β1) regulate the liver regeneration process by providing both stimulatory and inhibitory signals for cell proliferation. HGF will immediately stimulate the cell cycle and DNA synthesis when there are major changes in the complete mitogen expression for hepatocytes. PHx induces hepatocytes in the expression of a relatively large number of genes in the cell cycle (14), especially in extensive remodeling of the hepatic extracellular matrix (ECM), which occurs shortly after PHx. HGF is believed to play a primary role in liver regeneration by promoting cell proliferation, survival and morphogenesis through regulated DNA synthesis.

Materials and Methods

Animals and Ethanol (EtOH) Treatments

Male Spraque-Dawley rats were obtained from the National Science Council in Taiwan. Rats were acclimated for 1 week prior to the beginning of all experiments. Rats were oral administered with EtOH (5 ml/kg, 20%) one day once, for 6, 24, 72, and 168 h. Rats were obtained EtOH induced fibrotic liver rats and then performed 70% PHx for regeneration at 6, 24, 72, and 168 h, and then fed four TCMs (1 g/kg/day, CP, SMB, BK, and ESL) for 6, 24, 72, and 168 h to male Spraque-Dawley rats.

Experimental of the Fibrotic Rats PHx

Fibrotic livers induced by EtOH were subjected to 70% PHx. All of the surgical operations were performed the same. Ketamine was injected subcutane-

Table 1. The primer pair sequences were used by RT-PCR

Primers	Sequence	Tm	Length (bp)
Cyclin D1		45°C	480 bp
Forward primer	5'-AGGAGACCATTCCCCTGACT-3'		
Reverse primer	5'-TTCTTCCTCCACTTCCCCTT-3'		
Cyclin E		59°C	500 bp
Forward primer	5'-ACCTACAGTGAAGATGCACACC-3'		
Reverse primer	5'-CCTGTAGTTCTTGTTTCCTGCAC-3'		
pRb		48°C	470 bp
Forward primer	5'-AGGAGGACTGTTCTCTAAGG-3'		
Reverse primer	5'-GAGTGAGGTGTGTCTTCTGA-3'		
E2F		50°C	500 bp
Forward primer	5'-AACATCCAGAACATCCAGTGGGTAGGCAG-3'		
Reverse primer	5'-GGCTGTCAGTAGCCTCCAAG-3'		
MMP-2		60°C	450 bp
Forward primer	5'-CACCCCTGGCATCTTCTCCTT-3'		
Reverse primer	5'-AGCGTCTTCAGAGACAGCCAG-3'		
MMP-9		56°C	480 bp
Forward primer	5'-TAAGACTCACCTGGGTACTG-3'		
Reverse primer	5'-GCATGTAGTCACTCTTCACC-3'		
TGF-β1		45°C	450 bp
Forward primer	5'-ACAGCACGCTTGTGGAT-3'		
Reverse primer	5'-GTCTTCAAGCAAGAGGACCA-3'		
GAPDH		45°C	450 bp
Forward primer	5'-GGGTGTGAACCACGAGAAAT-3'		
Reverse primer	5'-CCACAGTCTTCTGAGTGGCA-3'		

ously at a dose of 30 mg/kg. The liver resections consisted of removing 70% of the fibrotic liver mass. The livers were collected at 6, 24, 72, and 168 h time points after the hepatectomy. The postoperative regenerating livers were excised and washed in PBS, then immediately frozen in liquid nitrogen.

Hot-Water Extract TCM Preparation

CP, SMB, BK, and ESL were extracted by boiling with distilled water for 1 h. The extraction was filtered, freeze-dried and kept at 4°C. The dried extract was dissolved in distilled water before use.

Western Blot

Remnant and regeneration proteins were separated using 12.5% SDS-PAGE and then transferred to PVDF. Membranes were blocked in 5% milk (diluted in Tris-buffered saline and 0.1% Tween 20) and incubated with the appropriate primary antibodies (HGF, focal adhesion kinase (FAK), Cyclin D1, Cyclin E, and retinoblastoma (pRb)) at 4°C overnight. HRP anti-IgG was used as the secondary reagent. After extensive washing the targeted proteins were detected using enhanced chemiluminescence (ECL).

Reverse Transcriptase PCR (RT-PCR)

For RT-PCR analysis, total RNA derived from liver homogenized tissues. The first-strand synthesis was applied according to the manufacturer's instructions. PCR primers were used as shown in Table 1. One μg of total RNA was performed with a reverse transcription reaction and reverse transcriptase. The cDNA was amplified using Tag DNA polymerase. The initial step was denatured at 95°C, 1 min then with 35 cycles of denaturation at 95°C for 30 s, annealed at 50-60°C for 40 s, and elongation at 72°C for 2 min. The final extension at 72°C for 10 min was applied to all reactions. The RT-PCR results were analyzed based on the following electrophoresis on 1.5% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide.

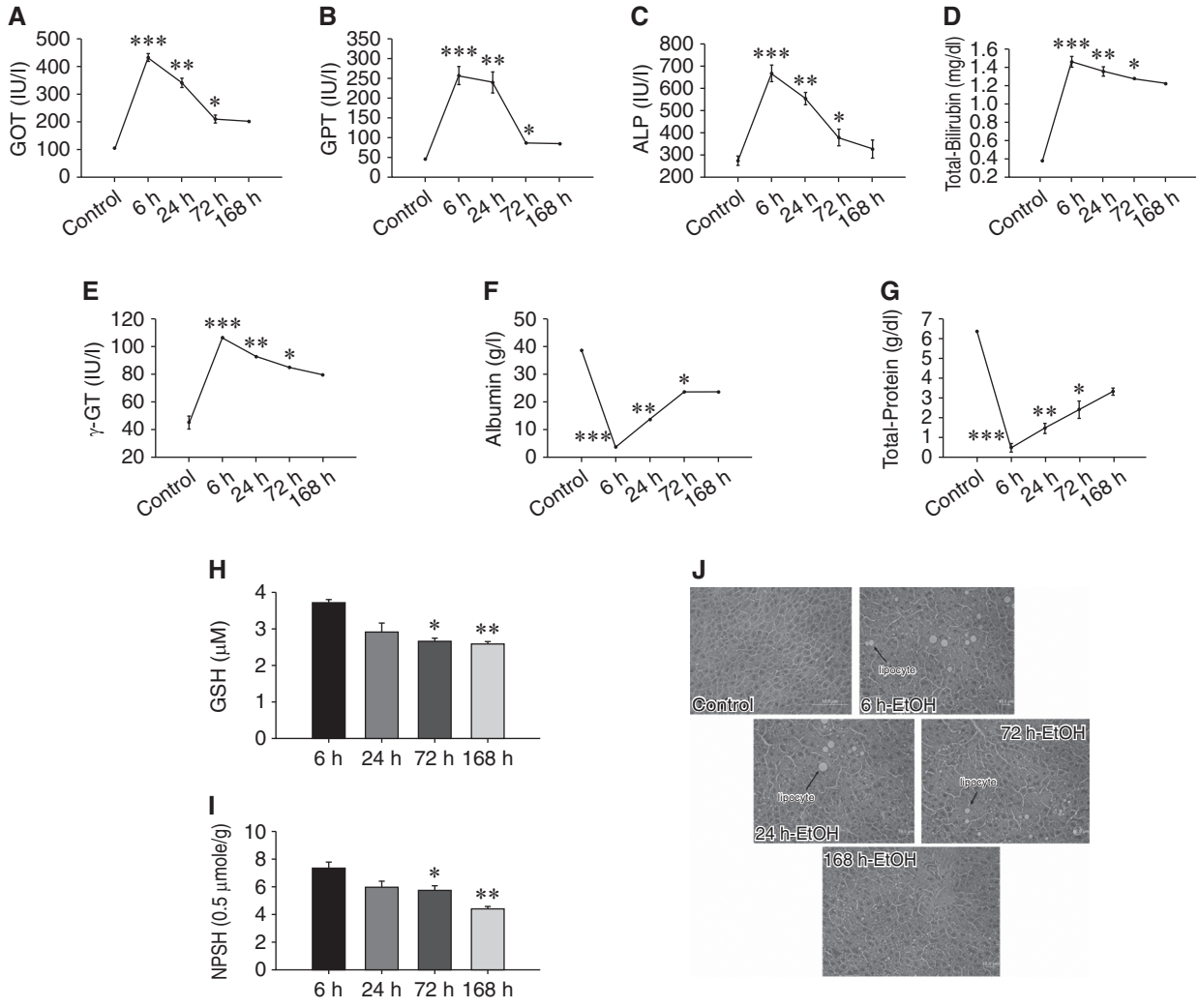

Fig. 1. Establishment of liver fibrosis in rats. Biochemistry function of liver. GOT (A). GPT (B). ALP (C). Total-Bilirubin (D). γ-GT (E). Albumin (F). Total-Protein (G). GSH (H). NPSH (I). Photographs of fibrotic rat livers (J). Quantification of densitometry analysis of protein levels. All data are presented as means ± SD (n = 6). In (A0-(G), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant difference compared with the corresponding control group. In H and I *$P < 0.05$, **$P < 0.01$, significant difference compared with the corresponding 6 h group.

Statistical Analysis

All data examined were expressed as mean ± standard deviation (SD). For Western blot and analysis, quantitation was carried out by scanning and analyzing the intensity of the hybridization signals using the FUJIFILM Imagine program. Statistical data analysis was performed using SigmaStat software. Comparison between groups was made using two way ANOVA test followed by the *post-hoc* test for equality of variances. A P value of less than 0.05 was considered to be statistically significant.

Results

Establishment of the Fibrotic Animal Model with EtOH Induced Hepatic Damage in Rats

EtOH (5 ml/kg) causes liver injury when administered to normal rats. Serum biochemical values were administered sterile saline (normal) and EtOH, results as Fig. 1. Rats receiving EtOH treatment exhibited a significant increase in some biomarkers and pathological findings. Elevations in glutamyl oxaloacetic transaminase (GOT), glutamyl pyrubic transaminase (GPT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γ-GT) and total-bilirubin activities and reductions in albumin and total-protein in serum were observed. Liver biological function markers, glutathione (GSH) and nonprotein sulfhydryl (NPSH), were also increased initially and then decreased at 72 ($P < 0.05$) and 168 h ($P < 0.01$). After treatment all bio-

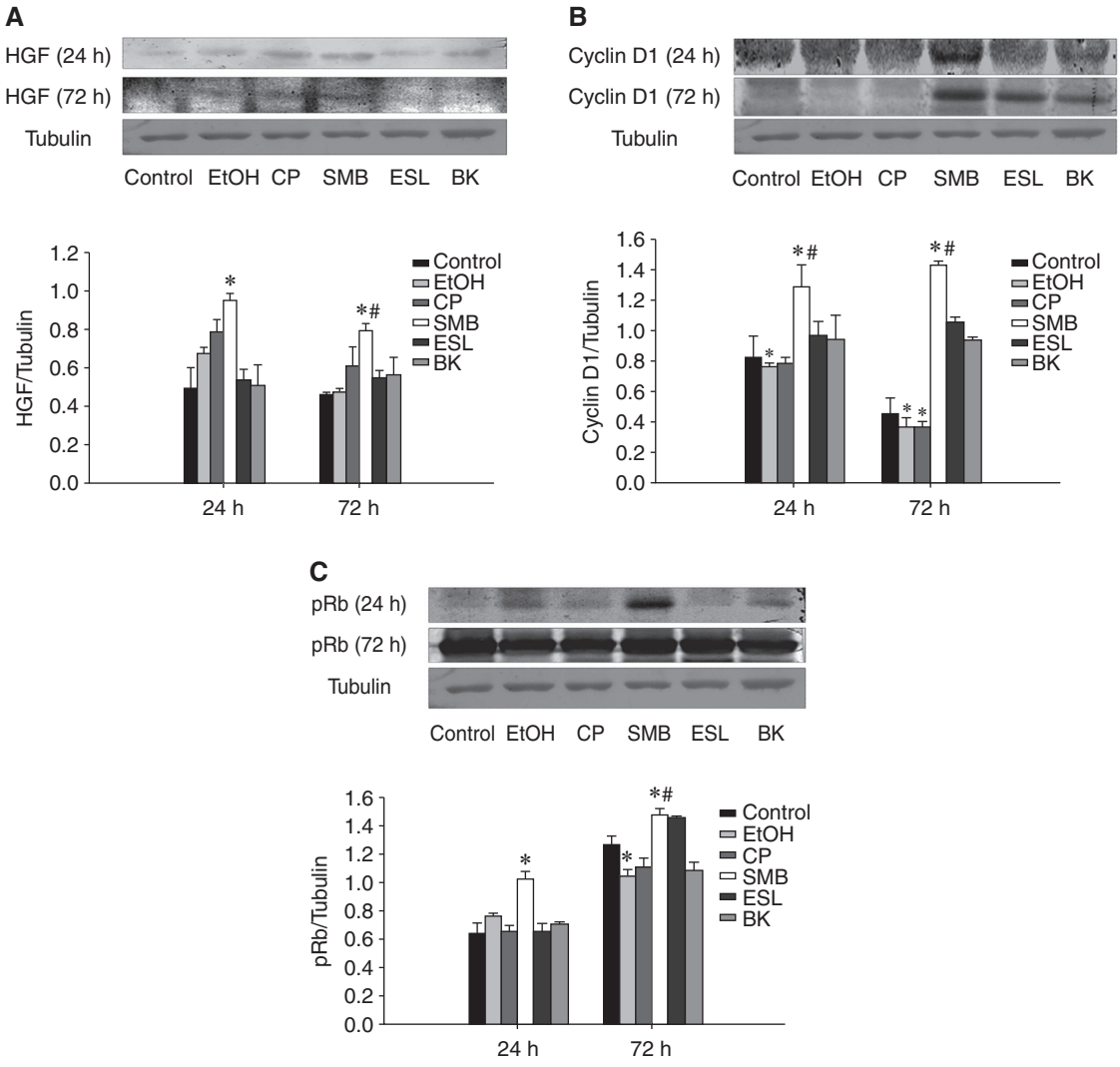

Fig. 2. A traditional Chinese medicine (TCM), SMB, effects on fibrotic rat after 24 and 72 h EtOH-treated. Western blotting analysis of HGF (A), Cyclin D1 (B), and pRb (C) expression levels by TCMs including CP, BK, ESL, and SMB. Equal amounts of lysate were separated by 12.5% SDS-PAGE. Quantification of densitometry analysis of protein levels. All data are presented as means ± SD (n = 6). $^{*}P < 0.05$, significant difference compared with the corresponding control group. $^{\#}P < 0.05$, significant difference compared with EtOH group.

logical markers increased temporarily at 6 h and then decreased until EtOH withdrawal. In contrast we also observed the histology and morphology of liver sections were disordered (Fig. 1J).

Fibrotic Rats Treated with TCMs Accelerate Liver Regeneration Signals

We detected the role of the TCM in the toxic injury process in rats. The molecular mechanisms of HGF, FAK, Cyclin D1, and pRb protein expression in TCM treatment after EtOH administration at 24 and 72 h were examined ($P < 0.05$). We found that the TCM, SMB, improved HGF, FAK, Cyclin D1, and pRb protein expression after EtOH administration toxicity injury at 24 and 72 h. Moreover, the results showed that HGF, FAK, Cyclin D1 and pRb expression declined after oral administration EtOH 24 and 72 h ($P < 0.05$) (Figs. 2 and 3). We also detected G_1 phase checkpoints, Cyclin D1, mRNA expression levels. Fig. 3A, shows that Cyclin D1 mRNA expression was also increased by SMB ($P < 0.05$). We determined that SMB protected against liver injury after EtOH administration at 24 and 72 h. It went without saying that HGF, FAK, Cyclin D1, Cyclin E and pRb expression decreased from EtOH injury. Interestingly, we did not find Cyclin E expression increased by SMB after EtOH injury at 24 and 72 h ($P < 0.05$). However, we could find that a TCM, ESL, induced Cyclin E expression after EtOH injury

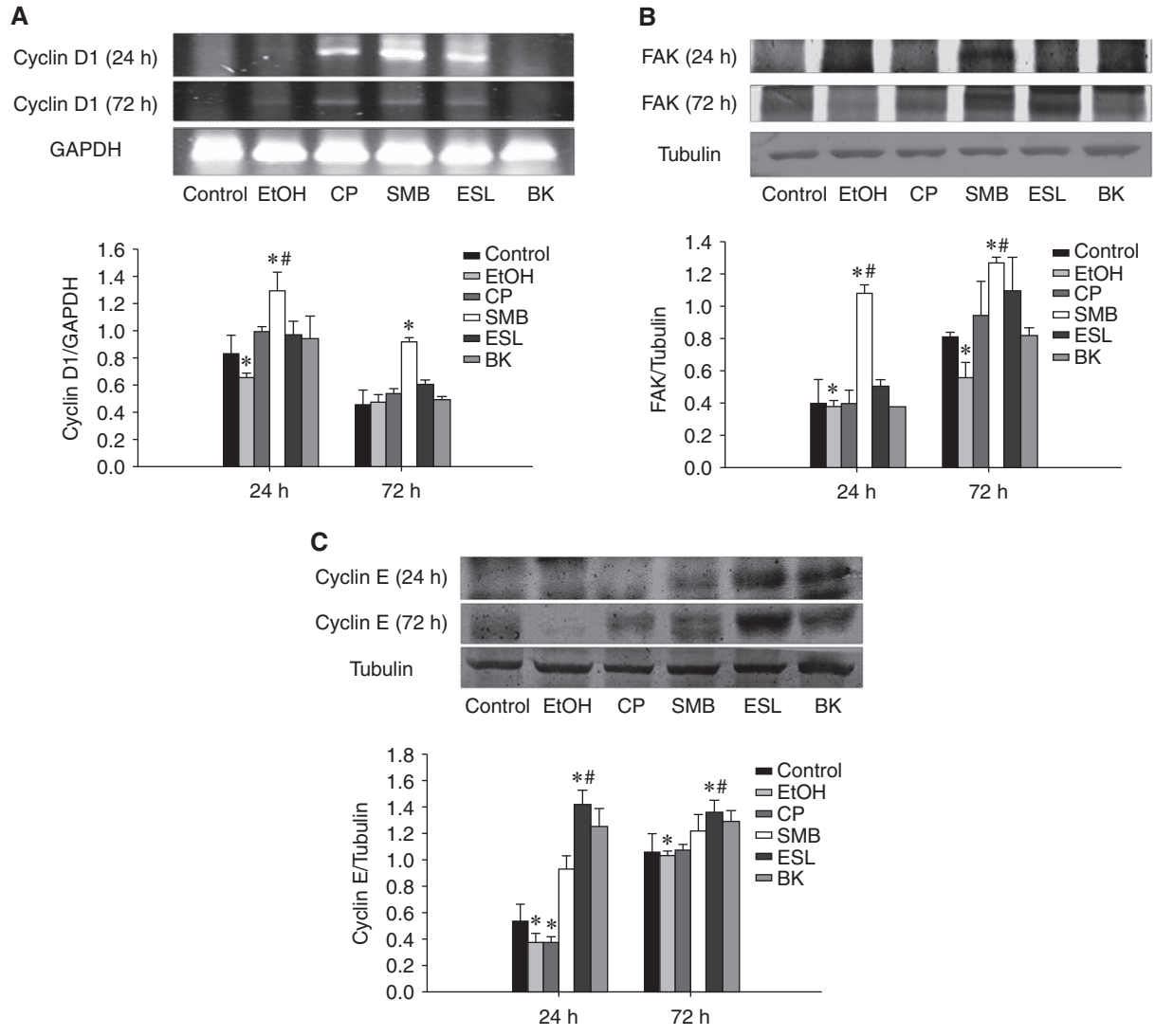

Fig. 3. TCMs, SMB and ESL, effects on fibrotic rat after 24 and 72 h post-EtOH treatment. RT-PCR analysis of Cyclin D1 mRNA expression by TCMs including CP, BK, ESL, and SMB (A). Western blotting analysis of FAK and Cyclin E expression levels by TCMs including CP, BK, ESL, and SMB (B). Equal amounts of lysate were separated by 12.5% SDS-PAGE. Quantification of protein levels densitometry analysis. All data are presented as means ± SD (n = 6). *$P < 0.05$, significant difference compared with the corresponding control group. #$P < 0.05$, significant difference compared with EtOH group.

at 24 and 72 h (Fig. 3C) ($P < 0.05$).

SMB Affords Protection against EtOH Induced ECM Degradation in Fibrotic Liver

At liver fibrosis onset increased matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) expression plays a major role in dynamic ECM modifications. We found that SMB reduced gelatinase, MMP-2 and MMP-9, mRNA activities after 24 and 72 h ($P < 0.05$) (Fig. 4A). MMP-2 and MMP-9 mRNA expression were increased by EtOH contributing to fibrosis ($P < 0.05$). However, we found that TIMPs mRNA activities were increased by CP ($P < 0.05$) after 24 h post EtOH treatment. It is important to note that CP induction by *Codonopsis pilosula* was greater than that produced by SMB (Fig. 4B) ($P < 0.05$).

Fibrotic Liver Regeneration after PHx

Rats subjected to PHx showed significant changes in hepatocyte architecture during the first 24 h after surgery (Fig. 5). However, liver regeneration from 24 to 168 h following PHx exhibited enlarged liver mass and liver regeneration index (Fig. 5, C and D). Fig. 5, shows that the liver regeneration index (%) increased at 24 ($P < 0.05$), 72 ($P < 0.01$), and 168 h PHx ($P < 0.001$) compared with 6 h post-surgery. Therefore, liver regeneration did occur after PHx, even in fibrotic or cirrhotic livers.

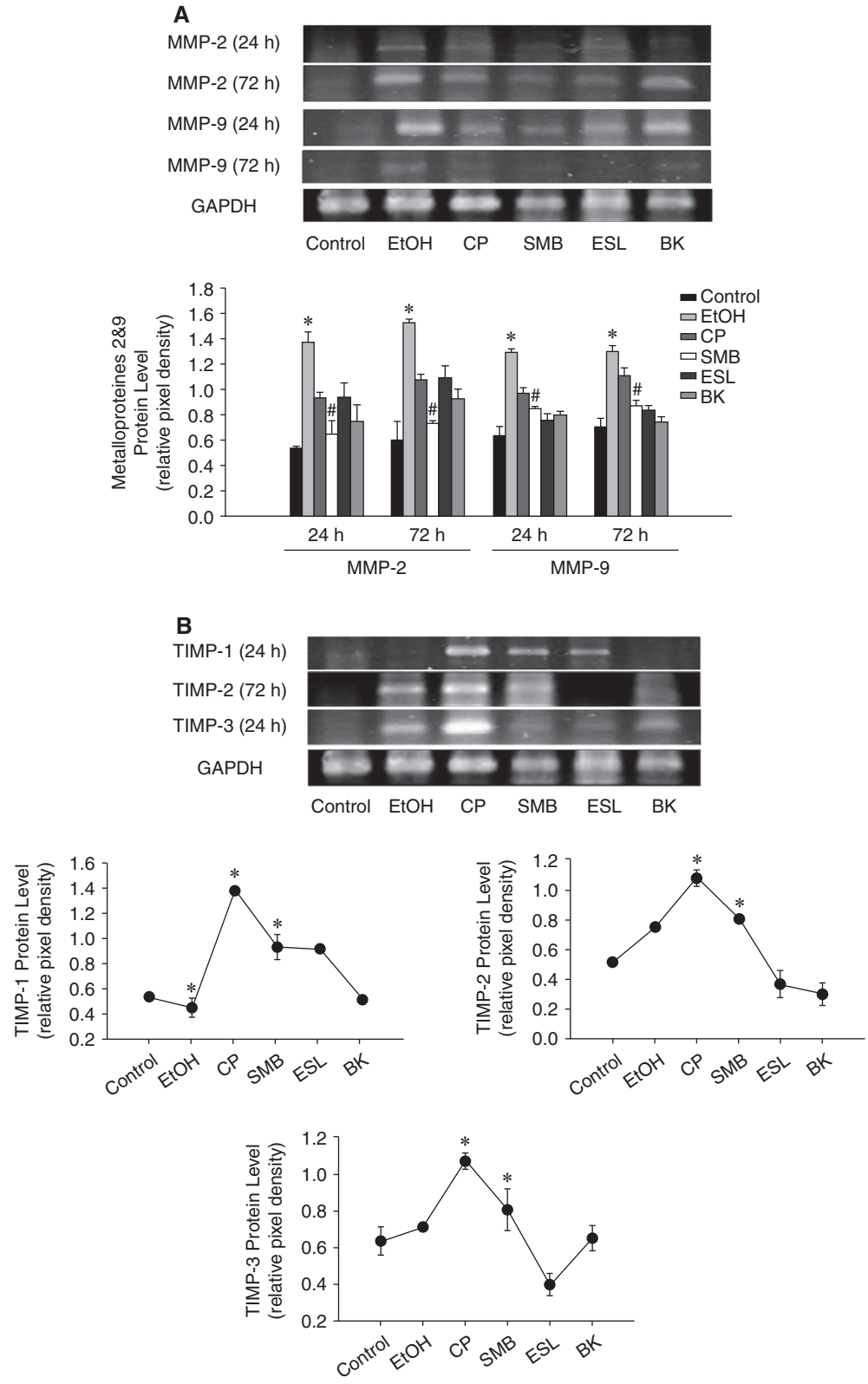

Fig. 4. TCMs effect on gelatinases, MMP-2 and MMP-9, and TIMPs protein amount in the fibrotic liver. RT-PCR analysis of MMP-2 and MMP-9 mRNA expression levels by TCMs including CP, BK, ESL, and SMB, after 24 h and 72 h EtOH-treated (A). RT-PCR analysis of TIMP-1, TIMP-2 and TIMP-3 mRNA expression levels by TCMs including CP, BK, ESL, and SMB after 24 h EtOH-treated (B). Equal amounts of lysate were separated by 12.5% SDS-PAGE. Quantification of densitometry analysis of protein levels. All data are presented as means ± SD (n = 6). $^{*}P < 0.05$, significant difference compared with the corresponding control group. $^{\#}P < 0.05$, significant difference compared with EtOH group.

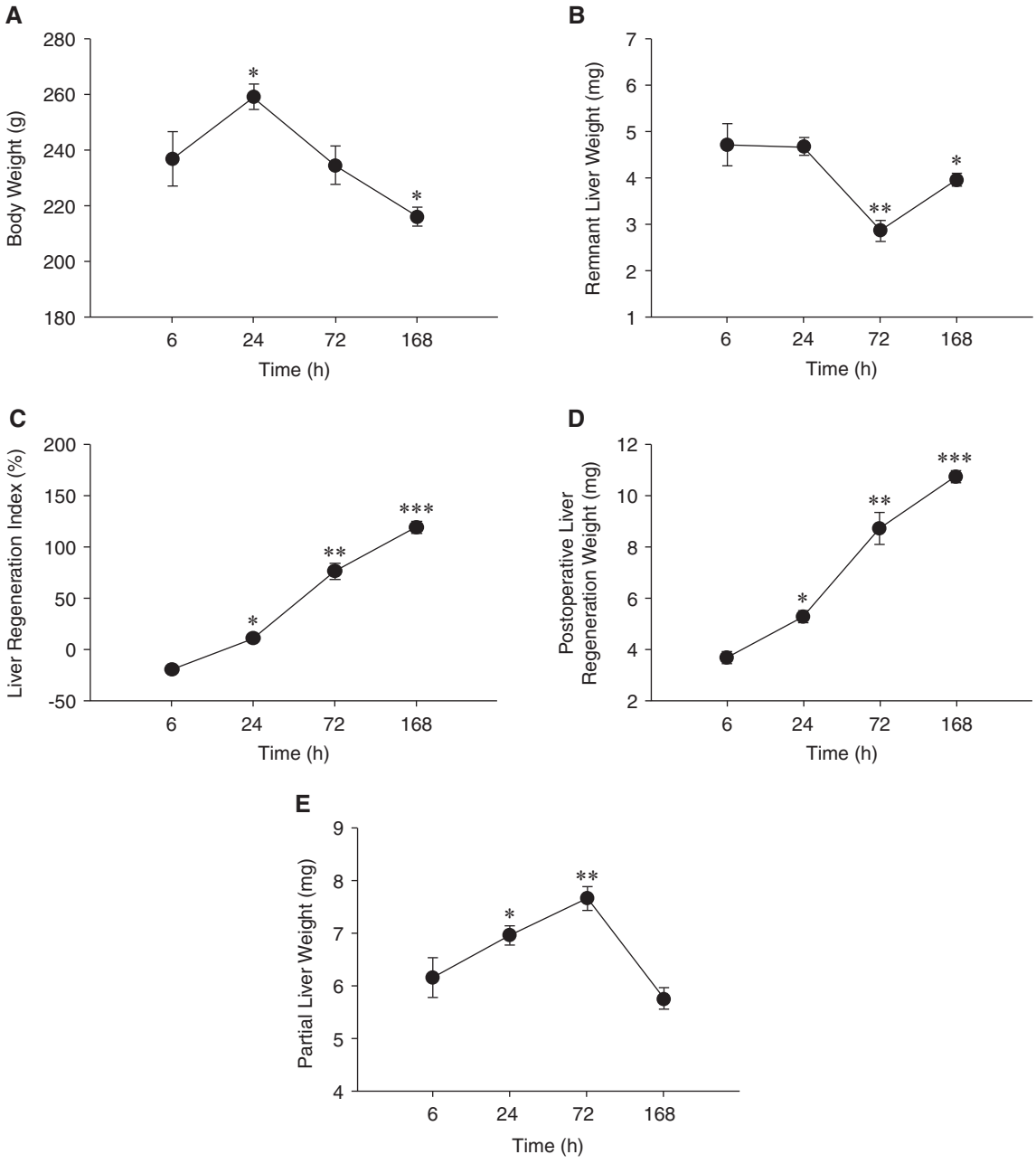

Fig. 5. Liver regeneration (%) in fibrotic rat. Body weight (A). Remnant liver weight (B). Liver regeneration index (%) (C). Postoperative liver regeneration weight (D). Partial Liver Weight (E). Quantification of densitometry analysis of protein levels. All data are presented as means ± SD (n = 6). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant difference compared with the corresponding control group.

ESL Induced Endogenous Cytokine, TGF-β1, mRNA Expression in Fibrotic Rats Liver Regeneration after PHx

During partial liver hepatectomy the organ attempts to repair the injury site by producing internal scar tissue as quickly as possible. ESL, a TCM, reduced TGF-β1 mRNA activity in fibrosis livers 24 to 168 h following PHx. SMB, however, showed no significant changes 24 to 168 h in fibrotic livers following PHx (Fig. 6A). We found that TGF-β1 mRNA expression increased 24 h after oral EtOH administration ($P < 0.05$) and 168 h following liver regeneration in fibrotic rats ($P < 0.01$).

ESL Induced DNA Synthase, Cyclin D1 mRNA Activity at 24 h Liver Fibrotic following PHx

We suggest that TCMs may act as a cell cycle progression agent to make primed cells progress

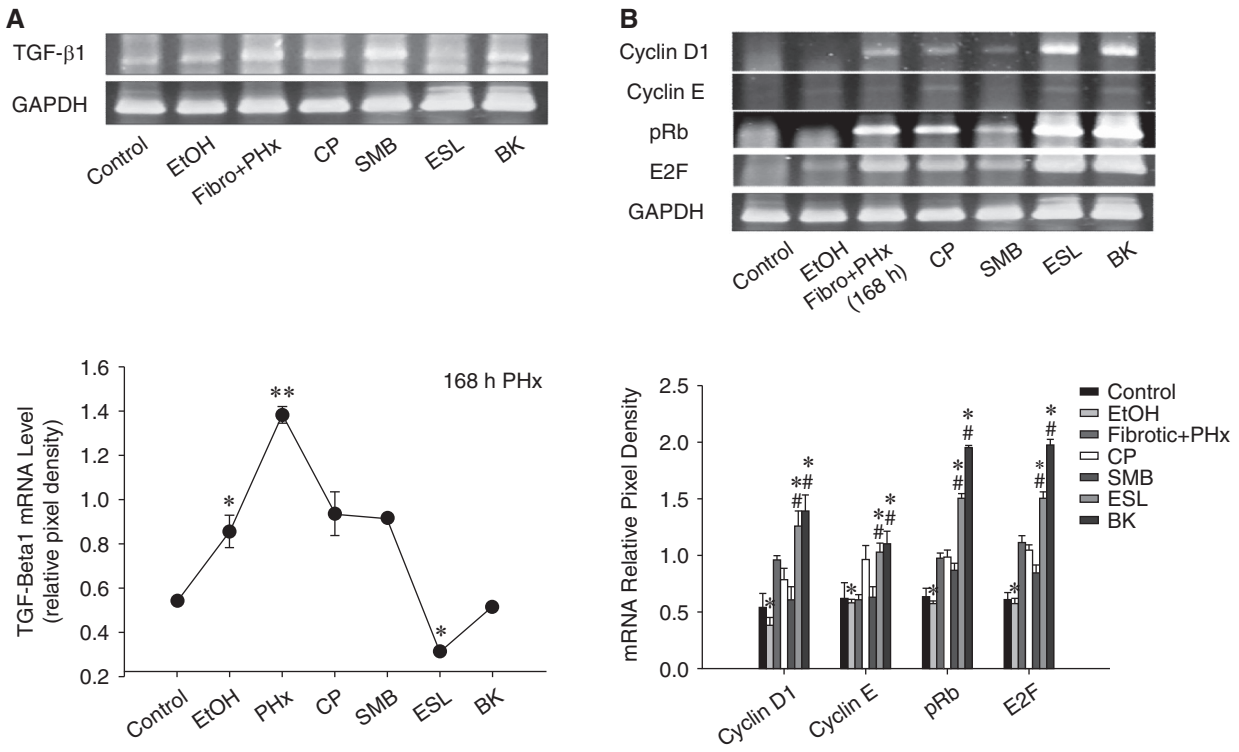

Fig. 6. After 24 h EtOH-toxic injury, TGF-β1 mRNA expression in liver fibrotic following PHx (A). After 24 h EtOH-toxic injury, Cyclin D1, Cyclin E, pRb and E2F in liver fibrotic following PHx (B). Equal amounts of lysate were separated by 12.5% SDS-PAGE. Quantification of densitometry analysis of protein levels. All data are presented as means ± SD (n = 6) $*P < 0.05$, $**P < 0.01$, significant difference compared with the corresponding control group. $^{\#}P < 0.05$, significant difference compared with EtOH group. CP, BK, ESL, SMB, SM.

through the cell cycle and DNA synthesis. In the S phase Cyclin D1, Cyclin E, pRb and E2F mRNA expression by RT-PCR were increased by ESL and BK after induced liver fibrosis (Fig. 6B) ($P < 0.05$). We also found a small increase in Fibrosis following PHx.

Discussion

Liver fibrosis is an alteration in chronic liver damage usually caused by alcohol and various toxins. Fibrosis to cirrhosis is the terminal stage of various liver diseases (15). However, the liver is one of the most complex organs with a potent orchestrated response regeneration capacity. In order to set the optimal mass in relationship to its liver functions the liver induces compensatory hyperplasia mechanisms (22). Previous reports indicted that herbal medicines have been used to treat liver disorders for thousands of years in the East and have now become a promising therapy internationally for pathological liver conditions (6). In the present study we presume that TCMs, CP, SMB, BK, and ESL may promote liver regeneration in fibrotic rats following PHx. We are interested in the effects of TCMs after surgical resection to remove a tumor together with surrounding liver cirrhotic or fibrosis tissue. We compiled and discuss the molecular biological analytical method of five herbal medicines for liver protection.

We observed that SMB induced HGF, FAK, Cyclin D1, and pRb protein expression in the G_1 phase after liver fibrotic injury at 24 and 72 h ($P < 0.05$) (Figs. 2 and 3). However, ESL induced cell cycle S phase liver regeneration after fibrotic injury. Most commonly, after the liver was injured it attempted to repair the injured site by producing internal scar tissue as quickly as possible (25). We could observe the liver producing ECM remodeling modification. Fig. 4, shows SMB prevented MMPs and TIMPs expression in fibrotic rats. Some previous research papers demonstrated that PHx creates a cell cycle dependent regulation and a potential physiological role in G_1 progression (10). However, we are interested in fibrotic liver tissue 24 to 168 h following PHx ($P < 0.05$). During this time the liver cell cycle is stronger than under normal conditions. After 168 h following PHx, alcohol toxicity subsides. We found that ESL and BK reduce TGF-β1 and enhance Cyclin D1, Cyclin E, pRb and E2F mRNA expression during liver regeneration in fibrotic rats (Fig. 6). We think that after 168 h PHx

the cell cycle transitions into the S phase. During this time different TCMs have different effects.

Liver cirrhosis fibrosis is a high risk factor for liver cancer. Surgery is the suggested treatment for hepatic tumor to remove the abnormal growth with the goal of preventing or arresting metastatic cancer (13). After surgery hepatocytes need to grow and maintain liver mass (21) and functions. We know that PHx hepatocytes proliferation function cannot maintain the integrated whole liver function (15). Drugs for hepatic growth liver regeneration have been used after or during PHx (2, 7). We suggest that TCMs may act as hepatoprotective agents in patients with hepatic carcinoma. It is worth noting that TCMs may act as hepatoprotective agents to accelerate cell cycle progress (25). Chinese medicine is currently the world's most effective treatment for liver disease (28). It has shown positive effects in treating nearly every known form of liver disease including cirrhosis (20), hepatitis, necrosis (3) and liver damage due to drug and alcohol abuse (14). That is why they are used today.

Acknowledgments

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