Original Paper

Anti-Fibrotic Effect of Plumbagin on CCl₄-Lesioned Rats

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Key Words

Hepatofibrosis • Plumbagin • Metabolism • Inflammation

Abstract

Background/Aims: Our previous studies have shown that plumbagin effectively inhibits hepatic stellate cell (HSC) proliferation. Thus, plumbagin-mediated anti-fibrotic effects *in vivo* merit further investigation. **Methods:** We used rat models to assess the potential benefits of plumbagin against CCl₄-induced liver fibrosis. **Results:** The results showed that plumbagin lowered the serum concentrations of liver functional enzymes (ALT, AST, ALB, TBIL) in CCl₄-fibrotic rats while reducing inflammatory cytokine levels (IL-6, TNF-α). As reflected in pathological examinations, rats that were administered plumbagin showed decreased collagen markers (HA, LN, PCIII and CIV) in liver tissues and improved hepatocellular impairments. In addition, plumbagin contributed to down-regulating NF-κB and TLR-4 mRNA in CCl₄-lesioned livers. As revealed in the immunohistochemical assay, plumbagin-administered rats showed reduced levels of α-SMA and TNF-α immunoreactive cells in liver tissue. **Conclusion:** Collectively, these findings offer appealing evidence that plumbagin may serve as an anti-fibrotic medication through inactivating the NF-κB/TLR-4 pathway that is associated with inflammatory reactions, thereby mitigating liver fibrosis.

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Introduction

Liver fibrosis is the result of excessive ECM accumulation, characterized by scar tissue replacement and regenerative nodules occurring in hepatic perisinusoidal space [1]. Fibrotic formation results from liver impairment, and its most common causes include hepatitis, alcoholism and other potentially damaging toxins [2]. Induced by inflammatory cytokines or mediators, activated stellate cells promote hepatic fibrosis and consequently disturb circulating blood flow in the liver. Physiologically, these pericytes secrete α -SMA that is responsible for connective tissue formation in response to liver injury, and α -SMA is commonly used as a marker of myofibroblast formation [3, 4]. Liver lesions from fibrosis are difficult to cure, but clinical interventions may block further development or reduce related complications [5]. However, current medications for managing hepatofibrosis are limited due to unwanted side effects [6]. Thus, developing a potential substitute medication that prevents liver fibrosis warrants further research. Applying TCM to treat hepatopathy is particularly common in China, possibly dating back centuries. More and more studies have documented that herbal extracts or plant-derived compounds are beneficial as alternative remedies to prevent liver fibrosis, such as tanshinone IIA [7], puerarin [8, 9] and Soshiho-tang formula [10]. Plumbagin is an active component originally isolated from *Plumbago zeylanica* L., which is characterized as having potent pharmacologic properties, including antimicrobial, anti-inflammatory, anticarcinogenic, immunosuppressive and anti-atherosclerotic properties [11-13]. More interestingly, our early studies have indicated that plumbagin causes cell cycle arrest and apoptosis in human HSC-LX2 cells by regulating the JAK2-STAT3 signal pathway and increasing apoptosis-associated protein expressions [14-16]. However, preclinical animal trials are needed to identify the efficacy of plumbagin before being recommended for medical prescribing. The present study was designed to explore the hepatoprotective benefits of plumbagin in a CCl_4 -induced fibrosis rat model and to discuss the possible molecular mechanism.

Materials and Methods

Materials

Plumbagin (purity>98.0%) was provided by the Physiological Department of Guangxi University of Traditional Chinese Medicine. Chemical grade CCl_4 was obtained from the Chengdu Kelong Chemical Reagent Factory (Chengdu, China). In addition, other reagents used were listed as follows.

Animals and plumbagin delivery

Healthy male SD rats that were one-month-old and weighed approximately 250 ± 10 g were obtained from the Medical Laboratory Animal Center of Guangxi Medical University, China (Using No. SCXK-Gui-2009-0002). This study was carried out through approved protocols of the Institutional Ethical Committee of Guangxi University of Traditional Chinese Medicine. Experimental rats were housed under controlled conditions at a temperature of $25 \pm 2^\circ\text{C}$, relative humidity of $60 \pm 10\%$, room air changes 12-18 times/h, a 12-h light/dark cycle and with access to food and water *ad libitum*.

The fibrosis rat preparation was conducted as previously described with some amendments [17]. Briefly, 80 male rats were randomly assigned into four groups ($n=20$, each group). The rats in the vehicle control group were intragastrically given the same volume of ethanol, and the rats in the CCl_4 control group was intragastrically given a 5% CCl_4 mixed peanut oil solution (0.5 ml/100 g) three times/week for eight weeks. The rats in the preparation-administered groups were gavaged with different doses of 4 mg/kg and 8 mg/kg plumbagin plus a 5% CCl_4 mixed peanut oil solution (0.5 ml/100 g) three times/week for eight weeks. The study was conducted in accordance with the U.S. guidelines (NIH publication #85-23, revised in 1985) for laboratory animal use and care.

Experimental samples

At the end of 8-week treatments, all of the rats were anesthetized with a 20% urethane (30 mg/kg intraperitoneal injection). Serum samples were collected into heparinized tubes (40 U/ml) for further use.

Liver specimens were removed and perfused with 4 °C saline. Part of the hepatic tissues was reserved at -80 °C for tests, whereas other specimens were fixed in 10% formalin for further histopathological inspection.

Liver index

Liver index= (hepatic weight/body weight) × 100%

Determination of serum enzymes and cytokines

Serological tests were used to measure the levels of ALT, AST, ALB, TBIL through the ultraviolet spectrophotometer at 480-520 nm followed by instructions from commercially available kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). In addition, plasma IL-6 and TNF-α contents were determined using an enzyme-linked immunosorbent assay according to the biochemical kit's protocols (Wuhan Boster Bio-Engineering Limited Company, Wuhan, China).

Measurement of collagen markers in the liver

Liver tissue was rinsed with a phosphate buffer three times and then was cryogenically homogenized with a Tris-HCl-EDTA (100 mM, pH 8.0) solution. Homogenate was collected after centrifuging at 8,000 × g for 15 min at 4°C. Intrahepatic concentrations of HA, LN, PCIII and CIV were determined using a radioimmunoassay following the manufacturer's instructions of available kits (Maker Science and Technology Co., Ltd., Sichuan, China). Final data are represented as ng/mg protein.

Histopathologic screening Liver-paraffin samples were sliced before they were subjected to routine hematoxylin-eosin and Masson's trichrome stains. The cell morphological alterations were screened and imaged using a light microscope (CX41-32C02, Olympus, Japan). Furthermore, the diagnostic standard of collagen deposition in the liver was evaluated as previously reported [18] as follows: grade 0, no fibrosis; grade 1, slight fibrosis; grade 2, mild fibrosis; grade 3, moderate fibrosis; and grade 4, severe fibrosis.

Ultrastructural changes of liver cells was checked using a transmission electron microscope (TEM) as has been previously described [19]. In brief, freshly prepared liver samples were fixed in pre-cold 2.5% glutaraldehyde + 4% paraformaldehyde phosphate buffer solution for 5 h. After washing the samples with phosphate buffer three times, the slice was extra-fixed with 1% osmium tetroxide for 3 h, dehydrated with stepwise ethanol solution, stained through a saturated solution of uranyl acetate containing 60% ethanol and embedded in araldite. Thus, the slices were scanned and imaged using an electron microscope at 80 kV (Hitachi, H-7650, Japan).

RT-PCR testing

Total RNA was extracted from liver specimens using a Trizol biochemical reagent (Tiangen Biochemical Technology Corporation, China). Target RNA purity was confirmed by spectrophotometer equipment before the reverse transcription of RNA-to-cDNA was conducted by the kit's protocols (Tiangen, Beijing, China). Real-time polymerase chain reaction was used to amplify the targeted DNA molecules followed by a 9700 PCR instrument (Bio-Rad, USA). Primer sequences in the liver samples are shown as follows: (NF-κB) forward primer: 5' AGT GTG GAG GCT GCC TTG CGA ATG 3'; antisense primer: 5' TGG GCT TTC AAG ACT GGA ACG GTC 3' (269 bp); (TLR-4) forward primer: 5' AGT TGA GGG GAC TTC CCA GGC 3'; antisense primer: 5' TCA ACT CCC CTG AAA GGG TCC G 3' (195 bp); β-actin sense primer: 5' CTG AGA GGG AAA TCG TGC CT 3'; and antisense primer: 5' CCA CAG GAT TCC ATA CCC AAG A 3' (208 bp). The PCR reaction procedure included 30 cycles using a general principle of a melting temperature of 96°C for 30 s, an annealing temperature of 70°C for 30 s and an extension temperature of 75°C for 10 min. Notably, β-actin was used as an internal control for the measurements. Thus, all of the target mRNAs were determined in a semi-quantitative manner using Opticon Monitor Software 3.1 (MJ, USA).

Immunohistochemical procedure

Liver slices were subjected to deparaffinization with different concentrations of dimethylbenzene and ethanol. After washing with buffered solution, hydrogen peroxide (3%, v/v) was added to the samples to deactivate the endogenous enzymes. After restoring the antigens, samples were exposed to 5% bovine serum albumin blocking solution for 5 min and then incubated with the primary antibodies and secondary antibodies (α-SMA, 1:600; TNF-α, 1:800, Santa Cruz, USA) for 1 h at 37°C prior to being added to a SABC

solution (Boster Bio-engineering Co. Ltd., China). Sections were detected with 3,3'-diaminobenzidine for a chromogenic reaction and counterstained the nucleus with hematoxylin. Subsequently, the final images from liver panels were captured through a light microscope (Carl Zeiss Inc., Germany).

Statistical analysis

Experiment-generated data were analyzed using SPSS 18.0 software (Chicago, IL, USA). Results were expressed as the mean $\pm$ SE. Differences between the groups were conducted through ANOVA with Bonferroni post-tests for the comparisons. The level of significance was set at $P < 0.05$.

Results

Plumbagin ameliorated the signs of CCl₄-injured rats

CCl₄-lesioned rats showed reduced body weights and elevated liver index ($P < 0.01$, $n=10$). Compared with the liver-injured rats, plumbagin-treated rats experienced notable improvements as shown in increased body weight and lowered liver indexes ($P < 0.01$, $n=10$) (Fig. 1A).

Plumbagin corrected the abnormal levels of serum enzymes and cytokines in CCl₄-injured rats

Compared with the vehicle control, CCl₄-injured rats resulted in a significant increase in serum contents of functional liver enzymes (ALT, AST, ALB, TBIL), whereas the inflammatory cytokine (IL-6, TNF- α) concentrations in plasma were elevated ($P < 0.01$, $n=10$). Interestingly,

Fig. 1. (A) Plumbagin increased the body weight and in turn reduced liver enlargement in CCl₄-fibrotic rats. (B) Plumbagin reversed aberrant serum concentrations of liver functional enzymes and inflammation cytokines in CCl₄-fibrotic rats. Data were analyzed using one-way ANOVA followed by Bonferroni post-tests, and the results are expressed as the mean $\pm$ SE ($n=10$). Notes: * $P < 0.01$ vs. vehicle control; ** $P < 0.01$ vs. CCl₄ control.

Fig. 2. (A) Plumbagin reduced the collagen accumulation in liver tissue of CCl₄-fibrotic rats. Fibrosis grades were assessed from all of the groups. Plumbagin reversed aberrant serum concentrations of liver functional enzymes and inflammation cytokines in CCl₄-injured rats. Data were analyzed through one-way ANOVA followed by Bonferroni post-tests, and the results were expressed as the mean $\pm$ SE (n=10). Notes: * P <0.01 vs. vehicle control; ** P <0.01 vs. CCl₄ control. (B) Histopathological examinations were conducted in the liver tissues from all groups (HE staining, scale bar: 100 μ m; Masson's trichrome stains scale bar: 100 μ m; TEM, scale bar: 2 μ m). The plus sign represents the central vein. Arrows represent the collagen-specific stain. The pentagram represents the inflammatory cell infiltration. Nu: nucleolus.

plumbagin reversed these abnormal changes, as was revealed in reductions of serum functional enzymes and inflammatory cytokines (P <0.01, n=10) (Fig. 1B).

Plumbagin attenuated the collagen deposition in the liver of CCl₄-induced fibrosis rats

As shown in Fig. 2A, CCl₄-fibrotic rats showed excessive productions of HA, LN, PCIII and CIV in liver tissue that were higher than levels in normal livers (P <0.01, n=10). Compared with fibrotic rats, these abnormal expressions of collagen markers were lower in the presence of plumbagin administration (P <0.01, n=10).

Histopathological detection

To screen for morphological alterations in liver cells, HE and Masson's trichrome stains were used for examination. The rats in the vehicle control showed intact lobular architecture accompanied with plenty of liver cells. Notably, the inflammation or collagen fibers around portal areas were undetected. Oppositely, CCl₄-lesioned liver exhibited signs of damage, such

Fig. 3. Plumbagin down-regulated the expressions of NF- κ B, TLR-4 mRNA in the liver at gene level (PCR assay). Data were analyzed through one-way ANOVA followed by Bonferroni post-tests, and the results are expressed as the mean $\pm$ SE (n=10). Notes: * P <0.01 vs. vehicle control; ** P <0.01 vs. CCl₄ control.

Fig. 4. Plumbagin decreased the immunoreactive numbers of α -SMA, TNF- α in liver tissue of CCl₄-fibrotic rats (immunohistochemical staining, scale bar: 100 μ m). Arrows represent the immunoreactive cells (brown). Data were analyzed through one-way ANOVA followed by Bonferroni post-tests, and the results are expressed as the mean $\pm$ SE (n=10). Notes: * P <0.01 vs. vehicle control; ** P <0.01 vs. CCl₄ control.

as cell lysis, vacuolation, inflammation or steatosis. In addition, widespread collagen fibers were disposed in hepatic lobules and liver sinusoid, resulting in increased fibrosis (Fig. 2A). As reflected in plumbagin-treated rats, liver tissue presented with significant ameliorations of repaired cytoskeleton, reduced inflammatory infiltration or cell regeneration, which were characterized by attenuated collagen accumulation in the liver and lowered fibrosis scores (Fig. 2A-B).

The ultrastructural examination from TEM highlighted that the normal liver architecture showed abundant organelles, such as mitochondria, endoplasmic reticulum, ribosome or intact nucleolus. In contrast, aberrant damage to the cytoskeleton or cell structures appeared in CCl₄-fibrotic rats, containing intralosomal mitochondria, oncotic endoplasmic reticulum, deformed nucleolus or well-established filaments associated with ECM. Interestingly,

plumbagin-administered rats exhibited signs of improvement, including repaired cell structure, increased organelles, reduced ECM deposit and emerged cell proliferation (Fig. 2B).

Plumbagin inhibited the expressions of NF- κ B, TLR-4 mRNA in the liver of CCl₄-induced fibrosis rats

To characterize signals of inflammation, two key regulatory genes in the liver were determined via PCR assay. The data showed that the liver from vehicle control indicated lower levels of NF- κ B and TLR-4 mRNA. Instead, CCl₄-injured liver resulted in remarkable up-regulation of NF- κ B and TLR-4 mRNA, in which the levels were higher than those in the vehicle control ($P < 0.01$, $n = 10$). In the presence of plumbagin administration, the results showed reduced expressions of these mRNAs in liver tissue ($P < 0.01$, $n = 10$) (Fig. 3).

Plumbagin reduced the immunoreactive counts of α -SMA and TNF- α in the liver of CCl₄-induced fibrosis rats

The analyses from the immunohistochemical staining showed that CCl₄-injured rats presented with increased counts of α -SMA (fibrogenic marker) and TNF- α (proinflammatory marker) immunoreactive cells in the liver compared with levels in the normal liver ($P < 0.01$, $n = 10$). Encouragingly, plumbagin-administered rats experienced reductions of endogenous α -SMA and TNF- α expressions in liver tissue ($P < 0.01$, $n = 10$) (Fig. 4).

Discussion

Hepatic fibrosis refers to the liver dysfunction characterized by excess ECM deposits. If left untreated, the lesion will cause life-threatening liver failure [20]. Many studies have suggested that fibrotic development can be controlled or inhibited through clinically therapeutic regimes [21]. In addition, plant-isolated active components, such as phloridzin [22], triterpenoid saponin [23] and puerarin [24], exert an effective antifibrotic role. Our data showed that body weight loss and hepatomegalia occurred in CCl₄-injured rats, indicating that the signs of malnourishment were due to CCl₄-induced hepatotoxicity. Instead, plumbagin mitigated liver enlargement, demonstrating that the benefits were associated with reduced liver toxicity and enhanced absorption of alimentary nutriment.

In clinical practice, liver function is tested through determining transaminase serum levels, in which high concentrations of these enzymes can reflect liver lesions [25]. Furthermore, cytokines may promote inflammatory development via a wide variety of cascade events. IL-6 release can relate to acute-phase of liver lesions associated with inflammation and may induce the release of other proinflammatory cytokines [26]. Interestingly, plumbagin inhibited these abnormal conditions that were consistent with mitigated liver injury, as shown in morphological observations. We preliminarily extrapolated that plumbagin-mediated hepatoprotection might benefit from anti-inflammatory action.

Pathologically, over-expression of ECM leads to liver fibrosis, in which other stress events aggravate hepatic damage, such as immune attack, and inflammatory infiltration [27]. Once the cell type of HSCs is activated, collagen or fibers would deposit in perisinusoidal spaces, progressively forming liver fibrosis [28]. Therefore, reducing the deposition of ECM can block fibrosis development. Our data suggested that CCl₄-fibrotic rats resulted in elevated contents of HA, LN, PCIII and CIV in liver tissue, characterized by embedded collagen fiber in the liver as shown in the Masson trichrome stain. Plumbagin corrected the abnormal collagen patterns in the liver, leading to speculations that one of the anti-fibrotic mechanisms of plumbagin is the inhibition of collagen formation via inactivating intrahepatic HSCs.

NF- κ B plays an important role in modulating the immune response to infection or stimuli [29]. Moreover, buildup of NF- κ B in liver cells can result in the recruitment of inflammatory cytokines/mediators, thus inducing fibrosis development [30]. TLR-4 exhibits a fundamental role in managing innate immunity activation and regulates the recruitment of

cytokines that are necessary for the progression of inflammation [31, 32]. Previous studies have found that high levels of TLR4 in the liver are implicated in inflammatory formation, thereby participating in fibrosis [33]. Hence, attenuating NF- κ B, TLR-4 expressions may inhibit the development of liver fibrosis. The present findings revealed that high levels of NF- κ B and TLR-4 in liver cells of CCl₄-fibrotic rats reflected liver impairments that are associated with inflammation. Interestingly, these profibrotic genes were down-regulated by administration of plumbagin. As a result, we postulated that the molecular mechanism against hepatofibrosis is linked to plumbagin-mediated inactivation of the NF- κ B/TLR-4 pathway, in which the benefit contributes to synergistic roles of attenuating immunotoxicity and inflammation stress in CCl₄-lesioned liver tissue, further correcting dysmetabolism to ameliorate liver functions.

As a fibrogenic marker, α -SMA is a pivotal protein that performs fibroproliferative processes in healing tissue, in which excessive expression of α -SMA can induce hepatofibrosis [34]. Evidence has indicated that there is a pronounced inflammation-associated correlation between α -SMA and TNF- α [35]. Thus, inhibiting α -SMA and TNF- α expressions contributes to the degradation of ECM for mitigating liver fibrosis. As a result, immunoreactive observations indicated that plumbagin decreased α -SMA and TNF- α protein expressions in liver cells, indicating that the benefits may be related to reducing hepatic collagen deposits, thereby improving liver metabolic functions.

Conclusions

In summary, our findings demonstrate that plumbagin may be an alternative anti-fibrotic medication due to its potential hepatoprotective effects. Although underlying mechanisms have been discussed, more in-depth studies need to be explored.

Abbreviation

HSC (hepatic stellate cell); CCl₄ (carbon tetrachloride); ALT (alanine aminotransferase); AST (aspartate aminotransferase); ALB (albumin); TBIL (total bilirubin); IL-6 (interleukin-6); HA (hyaluronic acid); LN (laminin); PCIII (type III procollagen); CIV (collagen, type IV); NF- κ B (nuclear factor-kappa B); TNF- α (tumor necrosis factor alpha); TLR-4 (Toll-like receptor 4); α -SMA (alpha smooth-muscle aorta); ECM (extracellular matrix); TCM (traditional Chinese medicine); SD (Sprague Dawley); SE (standard error); SPSS (statistical product and service solutions); ANOVA (one-way analysis of variance); HE (hematoxylin-eosin); TEM (transmission electron microscope); RT-PCR (reverse transcription-polymerase chain reaction).

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Disclosure Statement

None of the authors reports a duality of interest.

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