Alginate Suppresses Liver Fibrosis Through the Inhibition of Nuclear Factor-κB Signaling

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Purpose: Liver fibrosis (or liver scarring) is a causative factor for hepatitis, cirrhosis and hepatocellular carcinoma (HCC). Alginate (Agn) isolated from brown algae is known to slow the proliferation of fibroblasts, through the mechanisms of these effects remain undefined. This study explored the benefits of Agn on hepatic health and its associated mechanism(s) of action in hepatic stellate cells (HSC-T6s).

Materials and Methods: To assess the effects of Agn, HSC-T6s were treated with PDGF and cell proliferation, colony formation, cell migration, cell invasiveness and apoptosis were assessed. Rat models of liver fibrosis were produced through 12-week injections of intraperitoneal (IP) carbon tetrachloride (CCl₄). Rats were Agn-treated from weeks 8 to 12, and liver damage was assessed through Masson’s and H & E staining. Gene expression profiles were assayed via RT-PCR, Western blot and commercial ELISA kits.

Results: Agn reduced the proliferation of HSC-T6s and increased apoptotic rates through the downregulation of the Bcl-2:Bax ratio. Agn also inhibited the invasion and migration of HSC-T6s, prevented ECM deposition, and reduced the occurrence of liver fibrosis in rat models. Agn also prevented kBα and p65 phosphorylation.

Conclusion: Agn prevents liver fibrosis through its attenuation of HSC activation and division through the suppression of NF-κB in in vitro and animal models. This highlights how the clinical use of Agn can prevent hepatic fibrosis.

Keywords: liver fibrosis, cell proliferation, alginate, apoptosis, NF-κB

Introduction

Fibrosis is the first stage of liver scarring, which during later stages progresses to liver cirrhosis. Liver fibrosis encompasses a wound response to hepatic injury caused by autoimmune hepatitis, biliary obstruction, iron overload, nonalcoholic fatty liver disease, including nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH), viral hepatitis B and C, and alcoholic liver disease. The most common cause of liver fibrosis is nonalcoholic fatty liver disease (NAFLD), followed by alcoholic liver disease as a response to chronic alcohol abuse.¹ Liver fibrosis can be reversed through medical intervention² and the WHO reports that liver cirrhosis causes ~170,000 deaths in Europe alone each year.³ Elevated levels of extracellular matrix (ECM) deposition are a key feature of liver fibrosis. In the resting state, hepatic stellate cells (HSC) can be induced to form myofibroblasts that produce large levels of ECM.⁴ Inhibiting the division and activity of HSCs prevents extracellular matrix deposition and can reduce liver fibrosis.

Numerous molecules have protective effects on the liver. Antioxidants including vitamin E, lecithin and silymarin inhibit hepatocyte apoptosis and HSC activation,
Thus reducing liver fibrosis.\textsuperscript{5} Thiazolidinones as PPAR\(\gamma\) ligands, inhibit the activation of HSCs and play a protective role during hepatic fibrosis.\textsuperscript{6} Colchicine protects the liver mainly through anti-inflammatory effect.\textsuperscript{7} Other natural molecules can also treat liver fibrosis by inhibiting TGF-\(\beta\), NF-\(\kappa\)B and PI3K-AKT signaling pathways.

NF-\(\kappa\)B promotes hepatic damage, fibrosis and subsequent Hepatocellular Carcinoma (HCC).\textsuperscript{8} Blocking NF-\(\kappa\)B can increase HSC apoptosis and prevent liver damage.\textsuperscript{9} In unstimulated cells, NF-\(\kappa\)B dimerizes with I\(\kappa\)Ba, I\(\kappa\)B\(\beta\) and I\(\kappa\)B\(\gamma\) and is inactive. Upon I\(\kappa\)B degradation, NF-\(\kappa\)B is transcriptionally activated and binds DNA to enhance Bcl-2 transcription.\textsuperscript{10} NF-\(\kappa\)B is thus a critical regulator of HSC mediated liver fibrosis.

According to Traditional Chinese Medicine (TCM), the Liver is responsible for the smooth flow of emotions in addition to Qi and blood, and is a key target for therapeutic intervention. Alginate (Agn) is a water-soluble polysaccharide isolated from brown algae cytokernels that is a linear copolymer consisting of 1,4-L-glucuronic acid and 1,4-D-mannuronic acid.\textsuperscript{11,12} Since its discovery, Agn has been used for chemical, food and biochemistry applications.\textsuperscript{13–15} Due to its anti-viral,\textsuperscript{16} immune,\textsuperscript{17} anti-radiation,\textsuperscript{18} anti-coagulation,\textsuperscript{19} anti-oxidant,\textsuperscript{20} anticancer\textsuperscript{21} and lipid-lowering properties.\textsuperscript{22} Agn slows the proliferation of epithelial liver cells, cancer cells, and smooth muscle cells.\textsuperscript{21,23,24} Agn has been used for spleen and liver disease, but its effects on liver fibrosis remain largely undefined. In this study, we investigated the ability of Agn to prevent the growth and proliferation of HSCs and subsequent liver fibrosis.

\section*{Materials and Methods}

\subsection*{Chemicals}

PDGF-BB was purchased from PeproTech (Shanghai, China). Alginate (Agn, A0682) was obtained from Sigma (St Louis, United States). CCL\(_4\), ALT, LDH, Hyp and AST kits were purchased from the Nanjing Jiancheng Bioengineering (Nanjing, China). ELISA kits were purchased from Boyun Biotechnology (Shanghai, China). Primary antibodies were purchased from Cell Signaling Technologies (MA, USA). Western blot reagents were purchased from Beyotime (Shanghai, China). RT-PCR primers were purchased from Sangon Biotech (Shanghai, China). Other reagents were obtained from Takara Bio (Shiga, Japan).

\subsection*{HSC Culture}

HSC-T6 cells (Cell Bank of Chinese Academy of Sciences) were grown in DMEM supplemented with FBS (10\%) and Pen/strep (100 IU/mL) under standard tissue culture conditions (37\°C, 5\% CO\(_2\)).

\subsection*{Cell Viability and Proliferation Assays}

Viability was assessed via CCK-8 assays (Dojindo Laboratories Inc.). HSC-T6s in 96 wells (~8000 cells per well) were treated with Agn (12.5, 25, 50, 100, 200 or 400 \(\mu\)g/mL) in media for 48 h. All assays were performed in triplicate and normalized to no-drug controls. Cells were treated with CCK-8 for 2 h and absorbances were read at 450 nm. For proliferation assays, Agn or PDGF-BB (20 ng/mL) were added to HSC-T6s for 48 h, and CCK-8 assays were performed.

\subsection*{Cell Colony Formation Assays}

Cells were seeded into Petri dishes (60 mm, \(1 \times 10^3\) cells/dish) and Agn and/or PDGF-BB (20 ng/mL) treated as described. Media was replaced every 3-days and cells were fixed in PFA after 10 d of culture. Colonies were counted following crystal-violet staining (0.1\%).

\subsection*{LDH Assays}

HSC-T6 cells (\(8 \times 10^3\) cells/well) were drug treated and 60 \(\mu\)L of LDH solution was added at room temperature. Absorbances were read at 490 nm.

\subsection*{Flow Cytometry}

For apoptosis assessments, drug-treated HSC-T6s were EDTA treated and pelleted. Cells were resuspended and washed in 200 \(\mu\)L of binding buffer (Annexin V-FITC Staining kit, BD Biosciences) and Annexin-V-FITC/PI stained for 15 min. Samples were resuspended in fresh binding solution and apoptosis rates were assessed on a flow cytometer.

\subsection*{Cell Invasion Assessments}

HSC-T6s invasion was assessed in Transwells with 8-\(\mu\)m pore sized chambers. DMEM containing 20% FBS was added to the outside of the chambers and cells were seeded into inserts for 48 h (4\(\times 10^4\) cells/well) in DMEM plus 0.05\% FBS. Cells that had invaded were fixed in 4\% PFA, stained with crystal violet and counted.
Wound-Healing
Confluent HSC-T6s were wounded in culture plates using a p200 pipette tip followed by drug-treatments for 48 h. Migration into the scratch site was assessed at 0 h, 24 h and 48 h.

RT-PCR
HSC-T6 cultures (control and drug treated) were lysed in Trizol for RNA extraction, and the expression of collagen I and α-SMA mRNA were analyzed by RT-PCR. Values are relative to GAPDH and were assessed using the \(2^{-\Delta\Delta C_t}\) method. Primers: Acta2: for 5′-TGGCCACTGCTGCTCCTCTTCT-3′ and rev 5′-GAGGGCCAGCTTCTGCATACTCCT-3′; Col-Iα: for 5′-GGAGAGAGCATACGAGGATTG-3′ and rev 5′-GGGACTTCTTGAGGTTΔΔα-2-3′.

Western Blot
HSC-T6 cells or liver tissue were lysed, resolved via SDS-page electrophoresis and transferred to PVDF membranes. Membranes were incubated in 5% milk in TBST for 90 min at room temperature to block non-specific protein-protein interactions and labeled with the indicated antibodies at 4 °C overnight. The antibodies included Collagen I, α-SMA, Bcl-2, Bax, p65, IκBα, pp65, pIκBα and GAPDH (1:1000; Cell Signaling Technology, USA). Membranes were incubated with secondary antibodies for 1 h at room temperature and proteins were visualized using the commercial ECL system. Band intensities were quantified on Bio-Rad Image Lab 4.1.

In vivo Assessments
Animal protocols were approved by the Committee of Animal Care and Use at Wenzhou Medical University. (Number: wydw2019-0570). All experiments were performed ethically following the Guidelines for the Care and Use of laboratory Animals. Male SD rats weighing 200~220 g were purchased from the laboratory animal center of Wenzhou Medical University (Wenzhou, China). Models were acclimatized for 7-d during which free access to water and food and were provided in an air-conditioned facility at 22 ± 2 °C (12-h light). Body weights were assessed twice per week.

Eighty rats were randomly divided into 5 groups (n=16): (1) Normal; (2) CCl\(_4\); (3) CCl\(_4\) + Colchicine (Col) (0.2 mg/kg); (4) CCl\(_4\) + Agn (100 mg/kg); (5) CCl\(_4\) + Agn (200 mg/kg). Excluding the no-drug (normal) group, rats were treated through IP injections of CCl\(_4\) (0.2 mL/100 g, 1:1 in olive oil), twice per week over a 12 week period. Control groups received the same volume of olive oil alone. From weeks 8 to 12, rats in the Col-treated group were intragastrically administered 0.2 mg/kg Col per day, whilst the Agn group received intragastric 100 or 200 mg/kg doses. Rats in both normal and CCl\(_4\) groups received Saline. On the 12th week, serum and liver tissues were collected.

Serological Assays
Serum AST, ALT and hydroxyproline (Hyp) were assessed using commercial kits obtained from Nanjing Jiancheng Bioengineering. Assays were performed according to the described protocols.

ELISA Assays
IL-6, TNF-α, Laminin, type III precollagen (PCIII) and hyaluronic acid (HA) levels were assessed via ELISA (Shanghai Boyun Biological Technology).

Histology
For histological assessments, livers were fixed in PFA, paraffin embedded and sectioned (5 µm). Sections were H & E or Masson’s trichrome stained to visualize collagen deposition.

Immunohistochemistry
Sectioned tissues were deparaffinized and dehydrated using a gradient ethanol series. Sections were probed in retrieval solution for 25 min and microwaved. Sections were then cooled and blocked in 3% H\(_2\)O\(_2\). Sections were labeled with anti-Collagen I or anti-α-SMA primary antibodies (1:200) at 4°C overnight and stained with the indicated secondary antibodies.

Statistical Analysis
Data analysis was performed using SPSS20.0. Data are the mean ± SD. Shapiro–Wilk test together with box plots were used to assess data normality. The differences among multi-group was analyzed by one-way ANOVA followed by post hoc analyses using the Tukey’s test. Each experiment was performed in triplicate. P-values < 0.05 were deemed statistically significant.

Results
Agn Inhibits HSC-T6 Cell Proliferation
Figure 1 shows that in the presence of 12.5 to 400 µg/mL Agn for 48 h, cell viability decreased (p<0.001) (Figure 1A).
We selected 50, 100 or 200 µg/mL Agn for subsequent analysis and assessed its effects on HSC-T6 cell proliferation in the presence of PDGF-BB, a known stimulator of HSCs. Figure 1B shows that three different concentrations of Agn (p=0.0157<0.05, p=0.0015<0.01, p<0.001) inhibited PDGF-BB-induced (p=0.0034<0.01) cell growth. These effects were further confirmed by colony formation assays. PDGF-BB potently enhanced colony formation, which was attenuated by Agn (Figure 1C). These results confirmed that Agn inhibits the in vitro proliferation of HSC-T6 cells.

**Agn Induces HSC-T6 Apoptosis**

The loss of cell membrane integrity due to apoptosis or necrosis leads to LDH release into the culture medium. LDH therefore acts as a surrogate marker of cytotoxicity. We assessed HSC-T6 viability after Agn treatment ± PDGF-BB for 48 h. We found that Agn promoted LDH release and reduced HSC viability (p<0.001), PDGF-BB does the opposite (p<0.001) (Figure 2A). Cell apoptosis in response to Agn was assessed via Annexin-V-FITC/PI staining. Agn significantly increased apoptotic rates consistent with its detrimental effects on HSC viability (p<0.001) (Figure 2B and C).

As confirmation of these findings, Bel-2 and Bax (known indicators of apoptotic status) were assessed by Western blot in treated HSCs. The expression of Bel-2 decreased (p=0.0404<0.05, p<0.001, p<0.001) whilst the levels of Bax increased (p<0.001, p<0.001, p<0.001) in different concentrations of Agn treated HSC-T6 cells compared to PDGF-BB treated groups (Figure 2D–F).

**Agn Inhibits HSC Invasion and Migration**

Wound healing assays were used to assess the effects of Agn on HSC migration and motility. Agn potently inhibited HSC-T6 cell migration (p<0.001) (Figure 3A and B). Transwell assays were used to investigate cell invasion in response to Agn. Consistent with its effects on migration,
Agn treatment led to a concentration-dependent inhibition of HSC-T6 cell invasion (p<0.001) (Figure 3C and D).

**Agn Prevents HSC Activation and NF-κB Signaling in HSCs**

Collagen I and α-SMA are markers of HSC activation, both of which were significantly upregulated by PDGF-BB treatment (p<0.001). Importantly, these increases were attenuated by Agn (p<0.001) (Figure 4A–E).

NF-κB regulates apoptosis and cell proliferation. Figure 4C, F and G shows that NF-κB signaling decreased in response to Agn, which decreased the levels of p65 (Agn (50 µg/mL) groups versus PDGF-BB groups, p=0.0459<0.05, Agn (100 µg/mL) groups versus PDGF-BB groups, p=0.0013<0.01, Agn (200 µg/mL) groups versus PDGF-BB groups, p<0.001) and IκBα (Agn (50 µg/mL) groups versus PDGF-BB groups, p=0.0310<0.05, Agn (100 µg/mL) groups versus PDGF-BB groups, p<0.001, Agn (200 µg/mL) groups versus PDGF-BB groups, p<0.001) phosphorylation compared to PDGF-BB controls. These results suggest that Agn regulates the apoptosis and proliferation of HSCs through its effects on NF-κB signaling.

**Agn Suppresses CCl4-Induced Fibrosis and Liver Injury in vivo**

Serum AST and ALT are indicators of liver damage and were investigated to evaluate hepatic injury in vivo in response to Agn. ALT and AST levels significantly increased in CCl4 groups (p<0.001, p<0.001), whilst Agn
Agn inhibits HSC-T6 cell invasion and migration. Cells were treated as in Figures 1 and 2. (A) Wound healing assays. (B) Rates of wound healing. (C) Transwell assays. (D) Cell numbers. All experiments were repeated three times. ***P<0.01 and ****P<0.001 vs normal (no-drug groups); **P<0.01 and ***P<0.001 vs PDGF-BB.

Abbreviations: Agn, alginate; HSC, hepatic stellate cells; PDGF, platelet-derived growth factor.

(Agn (100 mg/kg) groups versus CCl4 groups, p<0.001, p=0.0296<0.05, Agn (200 mg/kg) groups versus CCl4 groups, p<0.001, p<0.001) and Col groups (p<0.001, p<0.001) showed a marked decrease in expression, confirming their protective effects in vivo (Figure 5A and B).

Serum Hyp is a biochemical marker of hepatic fibrosis, the levels of which significantly increased in CCl4 in vivo models (p<0.001) (Figure 5C). H & E staining revealed that an unordered liver structure, proliferation, fibrous connective tissue and inflammatory cell accumulation in the portal region compared to CCl4 treated groups (Figure 5D). Masson’s analysis revealed extensive collagen accumulation in CCl4 models (Figure 5E) which was alleviated through Agn and Col treatment. Fewer histopathological lesions and lower levels of liver fibrosis were also observed. Hyp levels similarly declined, confirming a protective effect.

**Agn Inhibits ECM Production in vivo**

Increased ECM deposition of collagen, fibronectin, laminin, and glycosaminoglycan occurs during liver fibrosis.\(^{25}\) We assessed ECM production through the tissue and plasma levels of \(\alpha\)-SMA, Collagen I, pCIII, LN and HA. \(\alpha\)-SMA and Collagen I expression significantly decreased in Agn (p<0.001, p<0.001) and Col (p<0.001, p<0.001, p<0.001) compared to CCl4 models (Figure 6A–C). The expression of the collagen biomarkers, pCIII, LN and HA also increased in CCl4 model groups (p<0.001, p<0.001, p<0.001). However, the increased levels of these biomarkers significantly decreased in response to Agn (Agn (100 mg/kg) groups versus CCl4 groups, p=0.0012<0.01, p=0.0130<0.05, p<0.001, Agn (200 mg/kg) groups versus CCl4 groups, p<0.001, p<0.001, p<0.001) and Col (p<0.001, p<0.001, p<0.001) (Figure 6D–F). IHC confirmed these findings (Figure 6G–H).

**Agn Inhibits NF-\(\kappa\)B Signaling and Inflammatory Cytokine Induced Apoptosis in Rats**

Figure 7A–C shows that CCl4 significantly upregulated pp65 and p\(\kappa\)B\(\alpha\) levels in liver tissue, whilst Agn or Col
remarkably reduced pp65 and pIκBα levels (p<0.001). NF-κB regulates many cellular genes/pathways including apoptosis, proliferation and Inflammation. As shown in Figure 7A, D and E, Agn (Agn (100 mg/kg) groups versus CCl4 groups, p=0.0403<0.05, p<0.001, Agn (200 mg/kg) groups versus CCl4 groups, p<0.001, p<0.001) and Col (p<0.001, p<0.001) significantly attenuated the induction of the anti-apoptotic protein Bcl-2, and increased Bax levels, confirming pro-apoptotic effects. Moreover, IL-6 and TNF-α levels increased in CCl4 groups, and sharply declined in response to Agn and Col (p<0.001) (Figure 7F and G). These data suggest that Agn prevents liver fibrosis through the downregulation of NF-κB signaling.

Discussion
Liver fibrosis encompasses the scarring of healthy liver tissue that results in a loss of liver function. Fibrosis represents the first stage of liver scarring, which during later stages progresses to liver cirrhosis. Liver fibrosis is commonly caused by autoimmune hepatitis, biliary obstruction, iron overload, nonalcoholic fatty liver disease, viral hepatitis B and C, and alcoholic liver disease. During disease progression, HSC activation plays a central role. In response to chronic liver injury, resting HSCs are activated and transdifferentiate into myofibroblasts that secrete high levels of ECM that includes elastin, collagen, glycoproteins, HA and proteoglycans.26 Preventing HSC activation therefore represents a promising treatment strategy.27 Agn is abundant and cost-friendly, and can inhibit smooth muscle cell proliferation and fibroblast mediated collagen synthesis.24,28,29 However, how Agn effects hepatic fibrosis has not been studied. We assessed the therapeutic effects of Agn in cultured HSCs and rat models of liver damage. We demonstrated that Agn significantly suppresses hepatic fibrosis in CCl4-injured rats and...
inhibit HSC activation through its effects on NF-κB signaling.

Col treatment in liver fibrosis patients leads to anti-fibrotic, anti-inflammatory and immunomodulatory effects. High-doses of Agn produced comparable anti-fibrotic effects. The IP injection of CCl₄ is classically used to simulate liver fibrosis. Serological assessments revealed a significant increase in serum ALT and AST activity in CCl₄ model groups, indicating serious hepato-cellular injury that was alleviated following Agn treatment. HE staining revealed severe histological damage in the liver tissues of CCl₄ model groups, but Agn treatment significantly attenuated these effects. Masson’s staining confirmed the high levels of collagen deposition in response to CCl₄, that could be reversed by Agn. These results demonstrate that Agn attenuates CCl₄ induced liver Injury. Agn reduced HSC proliferation and LDH release from treated HSC-T6 cells demonstrated the significant cytotoxic effects of Agn to HSCs. Apoptosis is a key to the prevention of liver fibrosis. To further examine the role of Agn during apoptosis, Bcl-2 and Bax expression were assessed in HSC-T6 cells and in in vivo rat models. The ratio of Bax:Bcl-2 determines apoptotic status. We found that Agn significantly increased the ratio of Bax: Bcl-2, which was a potent apoptotic compound.

Liver fibrogenesis increases ECM content including collagens I, III, and IV, fibronectin, undulin, elastin, laminin, hyaluronan and proteoglycans. PDGF-BB is a growth factor with multiple biological functions including the regulation of cell proliferation, viability, migration and connective tissue matrix synthesis including collagen, proteoglycans and glycosaminoglycans. These findings are consistent with previous studies in which α-SMA and collagen I expression were enhanced in PDGF-BB groups compared to untreated controls. Interestingly, Agn treatment significantly decreased α-SMA and collagen I levels in HSC-T6 cells. In addition,
Agn inhibited $\alpha$-SMA and collagen I expression in CCl$_4$-induced rats. The levels of pIII, LN and HA decreased after Agn treatment in CCl$_4$-induced rats assessed via ELISA. These results show that Agn prevents excessive ECM deposition both in vivo and in vitro.

Accumulating evidence implicates NF-$\kappa$B signaling as key to HSC proliferation. Aberrant NF-$\kappa$B activity also leads to liver fibrosis. Studies have demonstrated NF-$\kappa$B inhibition promotes HSC-T6 cell death via Bcl-2 suppression. Our finding were consistent with those of Jeong and colleagues who showed that Agn inhibits NF-$\kappa$B. We further showed that Agn inhibits p65 and IkB$\alpha$ phosphorylation. The expression of Bcl-2 also decreased, whilst Bax expression increased. Agn also inhibited NF-$\kappa$B nuclear translocation and the release of IL-6 and TNF-$\alpha$. These results confirmed that Agn reverses liver fibrosis through NF-$\kappa$B signaling.

Conclusions
We found that Agn inhibits HSC proliferation and migration and can prevent liver fibrosis through dampening NF-$\kappa$B signaling. These findings highlight the potential of Agn to alleviate liver fibrosis. Further studies in human subjects are now required.
Figure 7 Agn inhibits NF-kB mediated inflammation and signaling in vivo. Rats were treated as described in Figure 5. (A) Expression of p-p65, p65, p-IkBα, IkBα, Bcl-2 and Bax were investigated by WB. The levels of p-p65 (B) and p-IkBα (C) were quantified through normalization to p65 and IkBα. The relative expression of Bcl-2 (D) and Bax (E) were quantified by normalizing to GAPDH. IL-6 (F) and TNF-α (G) activity were investigated by ELISA assays. All experiments were repeated three times. *p<0.05 and ***p<0.001 vs normal (no-drug groups); #p<0.05 and ###p<0.001 vs CCl4-groups.

Abbreviations: Agn, alginic acid; NF-xB, Nuclear factor kappa B; CCl4, carbon tetrachloride; Col, Colchicine; IL, interleukin; TNF, tumor necrosis factor; ELISA, Enzyme-linked immunosorbent assay.

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Disclosure
The authors report no conflicts of interest in this work.

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