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World J Gastroenterol 2006 November 21; 12(43): 6926-6932
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Role of ethanol in the regulation of hepatic stellate cell function

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Received: 2006-07-18 Accepted: 2006-09-19

Abstract

Evidence has accumulated to suggest an important role of ethanol and/or its metabolites in the pathogenesis of alcohol-related liver disease. In this review, the fibrogenic effects of ethanol and its metabolites on hepatic stellate cells (HSCs) are discussed. In brief, ethanol interferes with retinoid metabolism and its signaling, induces the release of fibrogenic cytokines such as transforming growth factor β-1 (TGFβ-1) from HSCs, up-regulates the gene expression of collagen I and enhances type I collagen protein production by HSCs. Ethanol further perpetuates an activated HSC phenotype through extracellular matrix remodeling. The underlying pathophysiologic mechanisms by which ethanol exerts these pro-fibrogenic effects on HSCs are reviewed.

Key words: Ethanol; Acetaldehyde; Hepatic stellate cells; liver fibrosis; Type I collagen gene; Transcription factors; Transforming growth factor β-1

Wang JH, Batey RG, George J. Role of ethanol in the regulation of hepatic stellate cell function. *World J Gastroenterol* 2006; 12(43): 6926-6932

http://www.wjgnet.com/1007-9327/12/6926.asp

INTRODUCTION

Ethanol abuse is a leading cause for morbidity and mortality throughout the world. It affects many organ systems, most notably the liver causing both acute and chronic liver disease, and the central nervous system[1-3]. Hepatic cirrhosis resulting from alcohol abuse is one of the principal causes of liver-related morbidity and mortality. In the liver, excess ethanol leads to three pathologically distinct disorders, namely fatty liver (alcohol-associated hepatic steatosis), alcoholic hepatitis and cirrhosis. Alcohol-associated hepatic steatosis is the most common form of liver injury and is reversible with abstinence[3-5]. More serious forms of alcoholic liver disease (ALD) include alcoholic hepatitis characterized by persistent inflammation of the liver, and cirrhosis, characterized by progressive hepatic fibrosis. The pathogenesis of ALD is poorly understood, in part because no simple animal model exists that reproduces the full spectrum of the human disease, including the development of cirrhosis[1,4]. In addition, there is considerable variation among individuals in their susceptibility to ALD, so that among people drinking similar amounts, only a proportion develops cirrhosis[1,3-5].

Almost all ingested ethanol is metabolized in the liver. Two major enzyme systems, namely the oxidative and non-oxidative pathways, mediate the initial phase of ethanol metabolism[1,5] (Figure 1). The oxidative pathway comprises the alcohol dehydrogenases (ADH) and members of the cytochrome P450 system (predominantly CYP2E1)[5-7]. This pathway generates acetaldehyde. Acetaldehyde is subsequently metabolized to acetate via the mitochondrial enzyme acetaldehyde dehydrogenase (ALDH). Although acetaldehyde is oxidized to acetate by ALDH, the kinetics of this reaction is sufficiently slow to allow for the accumulation of acetaldehyde in humans or animals consuming alcohol[1,2,5]. The non-oxidative pathway of ethanol metabolism involves the esterification of ethanol with fatty acids to form fatty acid ethyl esters (FAEE), a reaction catalyzed by FAEE synthases[1,5].

Ethanol and its metabolites including acetaldehyde cause liver damage through several interrelated pathways[1,2,8,9]. The oxidation of ethanol is associated with a change in hepatocyte redox homeostasis which can lead to a number of metabolic disorders including lactic acidosis, hyperlipidaemia and hyperuricaemia. Chronic ethanol consumption does not influence ADH activity, but has a profound stimulatory effect on microsomal enzymes, particularly CYP2E1[1,2]. This is in part responsible for the development in alcoholic liver diseases, a rise in oxygen consumption, the excessive production of free radicals and an increase in the metabolism of ethanol, vitamin A and testosterone. Ethanol and acetaldehyde have deleterious effects both direct and indirect, for example by generating reactive oxygen species (ROS) and causing damage to

the intestinal mucosal barrier[1,10]. Cellular oxidative stress that is caused by the relative imbalance between free radical generation and insufficient anti-oxidant defense mechanisms, including reductions in glutathione, vitamin E and phosphatidylcholine, may be a principal mediator for the progression of alcoholic liver disease[1,2,10].

Steatosis, hepatitis and fibrosis seen in persons with ALD are a consequence of complex pathophysiological events involving various cell types within the liver including neutrophils, sinusoidal endothelial cells (SECs), Kupffer cells (KCs), hepatic stellate cells (HSCs) and hepatocytes. Recently, many studies have demonstrated that ethanol and its metabolites including acetaldehyde directly activate HSCs, the principal fibroblastic cell type within the liver[8,9,11]. Ethanol and acetaldehyde directly promote the production of transforming growth factor beta-1 (TGFβ-1) and several extracellular matrix (ECM) constituents including type I collagen by HSCs[8,9,11].

This article reviews recent advances in our knowledge on the effects of ethanol and its metabolites on HSCs.

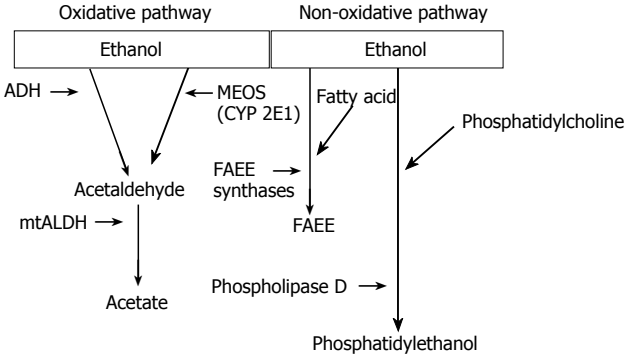

Figure 1 Metabolism of ethanol in the liver via oxidative and non-oxidative pathways. Oxidative pathway: In the first step of oxidation, ethanol is converted to acetaldehyde. Alcohol dehydrogenase (ADH) is the major enzyme. The microsomal ethanol-oxidizing system (MEOS) involves several cytochrome P450 proteins, of which cytochrome P450 2E1 (CYP2E1) is the major constituent. In the second oxidative step, acetaldehyde is rapidly metabolized to acetate by mitochondrial acetaldehyde dehydrogenase (mtALDH). Non-oxidative pathway: The non-oxidative pathway of ethanol metabolism involves the esterification of ethanol with fatty acids to form fatty acid ethyl esters (FAEE), a reaction catalyzed by FAEE synthases. The non-oxidative pathway also generates phosphatidylethanol *via* phospholipase D.

DIRECT EFFECTS OF ETHANOL ON HSCS

A central event in liver fibrosis is the activation of HSCs, which represents a transition from a quiescent vitamin A-rich cell type to a vitamin A-deficient, proliferative, fibrogenic and contractile myofibroblast. Activated HSCs demonstrate altered cell behaviors including proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, retinoid loss, leukocyte chemotaxis and cytokine release. In total, these changes result in excess ECM deposition which is reabsorbed, culminating in the development of liver fibrosis.

HSCs derived from the intragastric ethanol infusion model of ALD demonstrate an activated phenotype including an increase in collagen I and DNA synthesis[12], expression of α-smooth muscle actin (α-SMA) and depletion of retinyl palmitate[13].

Effect of ethanol on vitamin A metabolism within HSCs

HSCs are the major site of vitamin A storage in healthy adults. Vitamin A in HSCs is in the form of retinyl esters located in cytoplasmic lipid droplets[14]. The three active forms of vitamin A, namely retinol, retinal and retinoic acid (RA) are important regulators of cell proliferation and differentiation, binding to 2 distinct families of ligand-activated transcription factors: the retinoic acid receptor (RARs: RARα, RARβ and RARγ) and the retinoid X receptor (RXR)[15]. The natural ligand for the RARs is all trans-retinoic acid (ATRA). Published data indicate that HSCs from healthy rats express mRNAs in the RARs and RXRs[16].

Nutritionally reduced levels of serum and hepatic vitamin A have been reported in persons with ALD and in animal models of the disease[17,18]. In HSCs, ethanol significantly inhibits RA production[19] and reduces the retinol level[20]. Acetaldehyde exposure results in a reduction in RARβ message and protein in HSCs[21]. There are several other possible mechanisms by which ethanol can interfere with retinoid metabolism in the liver[19], including decreased vitamin A uptake, enhanced degradation of vitamin A in the liver, enhanced vitamin A mobilization from the liver to other organs, and degradation by ethanol of RA into polar inactive metabolites *via* induction of cytochrome P4502E1.

The activation and differentiation of HSCs are characterized by proliferation and an increase in the production of ECM proteins together with a loss of cellular retinoids. Therefore, it is plausible that ethanol-induced RA metabolism in HSCs could play a role in the development of alcohol-related liver fibrosis and cirrhosis.

Ethanol, HSC proliferation and α-SMA expression

Linolenic acid ethyl esters (LAEE), one of the FAEE products of non-oxidative ethanol metabolism, may promote HSC proliferation[22]. This effect is thought to be modulated through increased cyclin E and cyclin-dependant kinase 2 (CDK2) activities[22]. Ethanol, acetaldehyde and lactate, in contrast, have no direct effect on HSC proliferation[23,24].

Ethanol induces early protein expression of α-SMA in cultured HSCs compared to controls[25,26]. Chen and colleagues[27] likewise reported that α-SMA mRNA expression in HSCs is significantly enhanced by exposure to acetaldehyde. However, Poniachik *et al*[24] were unable to replicate this finding. Hence, the effects of ethanol on HSC proliferation and α-SMA expression remain controversial.

Effects of ethanol on ECM production by HSCs

HSC activation is characterized by an increase in the production of ECM, mainly collagen types I and Ⅲ. In addition, HSC activation is associated with alterations in both types of collagen, matrix-degrading metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs)[28]. Failure of matrix degradation leads to ECM accumulation and progressive hepatic fibrosis[28].

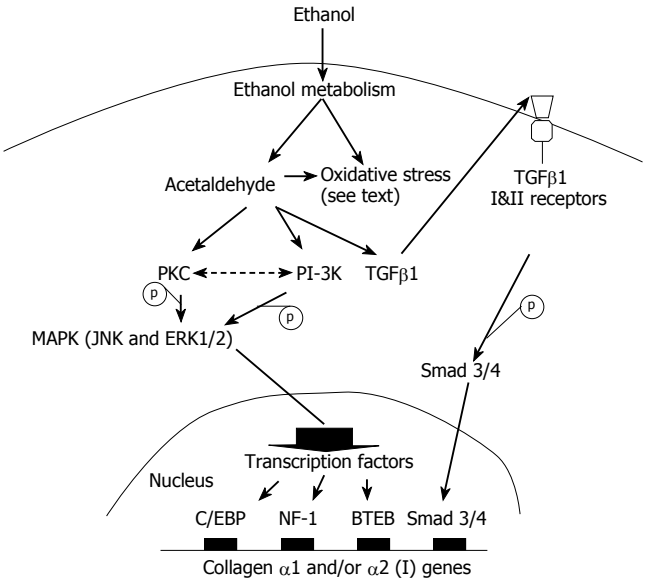

Figure 2 Possible mechanisms for the direct profibrotic effects of ethanol on hepatic stellate cells. Acetaldehyde, together with ethanol or acetaldehyde-derived oxidative stress, induces latent TGFβ1 and TGFβ receptor activation that subsequently leads to Smad3/4 activation and binding to the promoter of collagen I genes. PKC and/or PI-3K kinases are also activated by acetaldehyde. Both kinase systems activate their downstream components, including ERK1/2 and JNK. As a result, C/EBP, NF-1 and/or BTEB transcription factors are activated and therefore up-regulate collagen I gene transcription.

Numerous studies have shown that ethanol and/or its metabolites regulate the expression of multiple components of the ECM in HSCs. Both ethanol and acetaldehyde induce α1 (I) collagen mRNA expression in HSCs[29-33] but not hepatocytes[30]. This effect is protein synthesis-independent[29]. Acetaldehyde increases the steady-stage levels of α2 (I) gene expression[21,34] and the production of type I collagen protein[29-31] in HSCs. Acetaldehyde likewise up-regulates the mRNA expression of MMP-2 and fibronectin in human HSCs[35,36].

Signaling pathways that mediate type I collagen gene transcription in HSCs exposed to ethanol

The mechanisms by which ethanol and its metabolites regulate ECM gene and/or protein expression in HSCs have not been completely elucidated (Figure 2). Several centers have reported that the MAPK and PI-3K pathways are involved[35,37,38]. Anania and colleagues[37] noted that in rat HSCs, phospho-JNK is elevated following exposure to acetaldehyde. Inhibition of JNK by curcumin at low doses reduces acetaldehyde-induced steady-state levels of endogenous α1 (I) collagen mRNA expression[37]. Phosphorylated ERK and p38 are detectable but not significantly elevated. It seems likely therefore that JNK is the principal mediator of acetaldehyde-induced α1 (I) collagen gene up-regulation in rat HSCs. These finding are consistent with those previously reported by Chen *et al*[38]. In contrast, in human HSCs, ERK1/2 and the PI-3K pathway appear to be triggered by acetaldehyde, leading to α2 (I) collagen and fibronectin gene up-regulation[35].

The protein kinase C (PKC) pathway may also play a role in the up-regulation of collagen gene transcription following exposure to ethanol, since PKC is upstream of ERK1/2 and JNK[35,39,40]. Acetaldehyde-elicited α2 (I) collagen and fibronectin gene expression in human HSCs is inhibited by calphostin C (a PKC inhibitor). This PKC inhibitor also reduces the enhancing effect of acetaldehyde on α1 (I) collagen mRNA expression in cultured mouse and human HSCs[35,39]. Other experiments noted that acetaldehyde increases the translocation of PKC activity to membrane fractions[39] and both α1 (I) and α2 (I) collagen gene transcription in a calcium-independent manner[39].

Transcription factors that mediate type I collagen gene expression in response to ethanol

The modulation of gene expression in response to an exogenous or endogenous stimulus occurs through alterations in any one of the steps of gene transcription, mRNA stability, protein translation or protein degradation. Transcription factors are generally classified according to the conserved motifs within either their activation- or DNA- binding domains[41,42]. The binding of transcription factors at DNA-binding sites brings them into proximity with RNA polymerase Ⅱ and components of the transcription complex that assemble in the 5'untranscribed region of genes[42,42]. Transcription factors are then able to exert either a positive or a negative influence on the rate at which the transcription complex transcribes the gene of interest. Transcriptional control of acetaldehyde-induced type I collagen gene expression might be regulated through CCAAT/enhancer-binding proteins (C/EBP), nuclear factor-I (NF-I), basic transcription element binding (BTEB) protein as well as activating protein-1 (AP-1)[34,38,43,44]. The precise mechanisms however, remain to be clarified.

A C/EBP binding site is present in the α1 (I) collagen promoter between -365 and -335 of the transcription start site[44]. Transfection of the α1 (I) collagen promoter mutated at the C/EBP binding site results in unresponsiveness to acetaldehyde, indicating that this site is essential for the collagen gene transcription effect of acetaldehyde[34,44]. C/EBP consists of 6 members. The principal form present in activated HSCs is C/EBPβ[44]. In turn, four C/EBPβ isoforms with approximate molecular weights of 45, 43, 35 and 20 kDa have been identified in activated rat HSCs, with the 35-kDa isoform being predominant[44,45]. Attard *et al*[44] noted that activation of the α1 (I) collagen promoter by acetaldehyde in HSCs is most likely consequent upon an increase in this isoform and increased protein/DNA binding to the C/EBP binding site.

Another report suggests that acetaldehyde-induced α1 (I) gene expression in rat HSCs requires the binding of the acetaldehyde-inducible transcription factor BTEB to a GC box (-1484 to -1476) on the promoter of this gene[43]. In keeping with this proposal, blocking BTEB protein production, results in a reduction in acetaldehyde-induced α1 (I) collagen gene expression[43]. In an extension of the previous report, additional data suggest that acetaldehyde can firstly induce AP-1 activation in HSCs[38,43] and then the activated AP-1 can bind to AP-1 responsive elements in the BTEB promoter to stimulate BTEB expression. The BTEB protein, in turn, stimulates the expression of the α1 (I) gene in HSCs[38,43].

Nuclear factor I (NF-I), a CCAAT binding trans-

cription factor, is also known to bind to and activate the α1 (I) and α2 (I) collagen promoters[46]. Acetaldehyde-induced enhancement of the α2 (I) collagen promoter in activated HSCs is associated with increased binding of NF-I to a consensus consequence located at -352 to -104 bp from the transcriptional start site[34,46,47].

These data suggest that the transcription factors C/EBP, BTEB and NF-1 bind to and activate type I collagen gene transcription through each of them and/or *via* synergic effects, though further characterization of these effects is required. Whether these collagen gene transcription signaling pathways (after exposure to ethanol), are regulated by acetaldehyde itself, or in concert with other profibrogenic mediators such as oxidative stress or TGFβ1 is presently uncertain. The available data are discussed below.

Increased oxidative stress responses and TGFβ1 play an important role in the regulation of type I collagen gene transcription in HSCs

Oxidative stress: Increased oxidative stress is present in the liver after both acute and chronic ethanol administration[48]. Ethanol-induced oxidative stress within hepatocytes can occur acutely through ethanol metabolism or chronically following the induction of CYP2E1[2,10]. The oxidative metabolism of ethanol in hepatocytes elicits a range of mediators including ROS. CYP2E1 in particular has been shown to generate ROS including the superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyethyl free radicals[2,49]. Other sources of free radical generation by ethanol include NADH oxidation by aldehyde oxidase[50].

HSCs contain the enzymes of oxidative ethanol metabolism including ADH and P450 proteins[51-53]. Yamada and Oinonen[52] observed that CYP2E1 is present in rat HSCs as high as 21% of that found in hepatocytes. CYP2E1 is also detectable in the rat hepatic stellate cell line, HSC-T6[51]. In HSC-T6 cells overexpressing ethanol-inducible CYP2E1, time- and dose-dependent induction in collagen α2 (I) mRNA together with increased H_2O_2 production by ethanol has been observed. Antioxidants, including catalase (an H_2O_2 scavenger) prevent this increase in collagen α2 (I) mRNA expression[51]. Because ethanol can be oxidized to acetaldehyde by the peroxidative activity of catalase[54], this decrease in collagen α2 (I) expression by catalase suggests that ethanol-derived acetaldehyde is not responsible for this effect. Svegliati-Baroni *et al*[11] and Greenwel *et al*[35] have also provided evidence to support the concept that increases in mouse α1 (I) and human α2 (I) collagen gene expression in HSCs by acetaldehyde are linked to elevated H_2O_2 production. For example, acetaldehyde-elicited type I collagen gene expression can be blocked by the addition of catalase[11,35], and is in part, TGFβ1-independent[11]. It is known that H_2O_2 activates MAPK pathways[49] and this activity might enhance the binding of the down stream transcription factors to acetaldehyde-responsive elements within the type I collagen promoter. Likewise, leptin induces H_2O_2 production and contributes to TIMP-1 expression in HSCs[55]. Collectively, these data suggest that increased H_2O_2 generation during the metabolism of ethanol by HSCs might play a critical role in their activation.

TGFβ-1: Ethanol and acetaldehyde increase autocrine TGFβ1 expression in HSCs. In turn, TGFβ1 is able to up-regulate type I collagen gene expression[32,34,38]. Anania and colleagues[34] noted that the effects of acetaldehyde-induced TGFβ1 in the regulation of α2 (I) collagen gene expression are mediated by a factor or factors that bind to nuclear factor I (NF-I) consensus sequence located at the -352 to -104 region of the α2 (I) gene promoter. Acetaldehyde further increases the secretion of both latent and active forms of TGFβ1 in cultured rat HSCs[38], and induces the expression of the type Ⅱ TGFβ receptor which is required for all TGFβ-mediated signaling events[38]. In transient transfection experiments, the combination of TGFβ1 and acetaldehyde could result in greater activation of the mouse α2 (I) collagen promoter than either TGFβ1 or acetaldehyde alone[34]. Taken together, these observations suggest that TGFβ1 could play a key role in acetaldehyde-induced collagen I gene activation.

Chen *et al*[38,43] have noted that acetaldehyde stimulates latent TGFβ1 secretion and TGFβ type Ⅱ receptor gene expression. BTEB might be the principal transcription factor binding to the GC box of the type Ⅱ TGFβ receptor gene promoter[38]. The authors proposed a model wherein acetaldehyde activates signal transduction pathways including PKC, JNK and ERK, leading to activation of AP-1. AP-1 is proposed to activate the gene expression of BTEB. BTEB then up-regulates TGFβ type Ⅱ receptor gene expression in HSCs. By stimulating latent TGFβ1 activation and secretion, as well as up-regulating the expression of TGFβ type Ⅱ receptor, acetaldehyde activates TGFβ1 signaling, which eventually enhances expression of the α1 (I) collagen gene in HSCs[38].

The precise molecular mechanisms by which acetaldehyde elicits TGFβ1 production in HSCs are largely unknown. Acetaldehyde might directly bind to the TGFβ1 gene promoter leading to its activation. Alternatively, acetaldehyde might bind to other gene promoters of transcription factors that in turn activate the TGFβ1 gene.

OTHER DIRECT FIBROGENIC EFFECTS OF ETHANOL ON HSCS

Rodriguez-Fragoso and his colleagues[56] investigated the effects of the activity of urokinase type plasminogen activator (uPA) in the CFSC-2G stellate cell line and demonstrated that acetaldehyde (175, 250 and 350 μmol/L) enhances uPA gene expression. This is accompanied with a concomitant increase in production of type I collagen. uPA plays an important role in matrix remodeling under a wide range of physiological and pathological conditions, activates TGFβ1 and induces proliferation of HSCs[57,58]. Furthermore, profibrogenic mediators including IL-6, TNF-α, malondialdehyde (MDA) and intracellular GSSG have been reported to increase in CFSC-2G cells treated with ethanol or acetaldehyde[59-61].

Malondialdehyde-acetaldehyde (MAA) -protein adducts induce a dose- and time-dependent increase in the secretion of chemokines including monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-2 as well as an increase in

the production and expression of intercellular adhesion molecule-1 (ICAM-1) in activated rat HSCs[62,63]. These effects may contribute further to the activation of HSCs and the subsequent development of alcohol-associated liver fibrosis.

ACTIVITY OF ETHANOL/ACETALDEHYDE ON PANCREATIC STELLATE CELLS

Rat pancreatic stellate cells (PSCs) exhibit features similar to those of HSCs[64-66]. These cells are abundant in alcoholic chronic pancreatitis in humans, suggesting a central role of this cell type in pancreatic fibrosis[65]. An effect of ethanol on the modulation of PSCs has been documented. Ethanol and acetaldehyde increase α-SMA protein and type I collagen synthesis in PSCs[67], likewise enhance PSC MMP-2 and TIMP-2 gene expression as well as TIMP-2 protein secretion[67-69]. Both ethanol and acetaldehyde increase the activation of all 3 subfamilies (ERK1/2, JNK/SAPK and p38 kinase) of the MAPK pathway in PSCs. Only p38 MAPK is responsible however, for the induction of α-SMA and α1 (I) collagen gene expression[70]. Moreover, ethanol and acetaldehyde-induced MAPK activation can be blocked by the antioxidant N-acetyl-cysteine, suggesting a role of oxidative stress in signal transduction[68,71].

CONCLUSION

Ethanol can be metabolized in hepatocytes and stellate cells to generate acetaldehyde and other metabolites. Ethanol and/or its metabolites including acetaldehyde have direct effects on HSC activation. These effects might be mediated by ethanol/acetaldehyde and/or ethanol/acetaldehyde-induced oxidative stress and TGFβ1 expression which activate relevant signaling pathways leading to the binding of transcription factors to the type I collagen gene promoter (Figure 2). As a result, ethanol augments the production of extracellular matrix proteins. Ethanol also stimulates the production of other profibrotic mediators, including IL-6, TNF-α and uPA. Taken together, these effects of ethanol/acetaldehyde on HSCs play an important role in the development of alcohol-associated liver fibrosis. Characterization of the key genes initiating and perpetuating the process of HSC activation by ethanol helps to further elucidate the molecular mechanisms of alcohol-associated liver fibrosis. In the future, it is hoped that specific, directed pharmacological agents can be selected and/or developed that target these mechanisms and thereby prevent or retard the fibrogenesis induced by alcohol.

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S- Editor Wang J **L- Editor** Wang XL **E- Editor** Ma WH