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Original Article

FGF21 treatment ameliorates alcoholic fatty liver through activation of AMPK-SIRT1 pathway

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Fibroblast growth factor 21 (FGF21), a recently identified member of the FGF superfamily, is mainly secreted from the liver and adipose tissues and plays an important role in improving metabolic syndrome and homeostasis. The aim of this study is to evaluate the role of FGF21 in alcoholic fatty liver disease (AFLD) and to determine if it has a therapeutic effect on AFLD. In this paper, we tested the effect of FGF21 on alcohol-induced liver injury in a murine model of chronic ethanol gavage and alcohol-treated HepG2 cells. Male KM mice received single dose of 5 g/kg ethanol gavage every day for 6 weeks, which induced significant fatty liver and liver injury. The alcohol-induced fatty liver cell model was achieved by adding ethanol into the medium of HepG2 cell cultures at a final concentration of 75 mM for 9 days. Results showed that treatment with recombinant FGF21 ameliorated alcoholic fatty liver and liver injury both in a murine model of chronic ethanol gavage and alcohol-treated HepG2 cells. In addition, FGF21 treatment down-regulated the hepatic expression of fatty acid synthetic key enzyme, activated hepatic AMPK-SIRT1 pathway and significantly down-regulated hepatic oxidative stress protein. Taken together, FGF21 corrects multiple metabolic parameters of AFLD *in vitro* and *in vivo* by activation of the AMPK-SIRT1 pathway.

Keywords alcoholic fatty liver; fibroblast growth factor 21 (FGF21); AMPK; SIRT1

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Introduction

Hepatic fat accumulation as a result of chronic alcohol consumption can induce liver injury. In the initial stage of alcohol-induced fatty liver disease (AFLD), triglyceride (TG) accumulates in hepatocytes inducing fatty liver (steatosis), although this process is reversible at this stage [1]. However, with continuing alcohol consumption, simple fatty liver can progress to more severe forms of liver injury such as alcoholic hepatitis, cirrhosis, and hepatocellular carcinoma [2]. Unfortunately, there are no specific medical treatments for patients with alcoholic fatty liver, thus, it is crucial to develop specific pharmacological drugs to treat alcoholic steatosis during the early stage of AFLD and prevent the progression to more severe forms of liver damage.

AMP-activated protein kinase (AMPK) is a master regulator of metabolism that senses cellular stresses (such as oxidative stress and reduced energy charge), increases the activity of the major energy-generating pathways (glycolysis and fatty acid oxidation), and down-regulates energy-demanding processes (fatty acid and cholesterol synthesis, protein synthesis) [3]. AMPK is a heterotrimer consisting of a catalytic subunit (AMPKα, with two isoforms α1 and α2), a scaffolding subunit (AMPKβ, with two isoforms β1 and β2), and a regulatory subunit involved in nucleotide binding (AMPKγ, with three different isoforms γ1, γ2, and γ3) [4]. Activation of AMPK increases fatty acid oxidation and inhibits synthesis, whereas inhibition of AMPK blocks fatty acid oxidation and promotes fatty acid synthesis [3]. In ethanol-fed rodents, AMPK activity was decreased [4,5], suggesting that ethanol's effects on AMPK appear to play a significant role in the pathogenesis of alcoholic fatty liver disease (AFLD).

Fibroblast growth factor 21 (FGF21), belonging to the fibroblast growth factor superfamily, is recently discovered as a new protein involved in glucose homeostasis, lipid metabolism, and energy balance [6]. FGF21 is preferentially expressed in tissues related to metabolic functions, such as liver and pancreas, and secreted following the agonist

effect of free fatty acids (FFAs) on peroxisome proliferator-activated receptor nuclear receptor alpha (PPARα) [7]. Our previous study showed that exogenous FGF21 can inhibit lipid synthesis, reduce hepatic TG accumulation, enhance glucose metabolism, and inhibit gluconeogenesis in the liver, and can thus reverse hepatic steatosis and fibrosis and prevent non-AFLD (NAFLD) [8]. Additionally, FGF21 regulates energy homeostasis in adipocytes through activation of AMPK and sirtuin 1 (SIRT1), resulting in enhanced mitochondrial oxidative function [9]. Thus, FGF21 has the potential to become a powerful therapeutic to treat alcohol-induced fatty liver disease.

In this study, the effects of FGF21 on AFLD were explored and whether the therapeutic effects are mediated through the activation of AMPK-SIRT1 pathway was also investigated.

Materials and Methods

Reagents and antibodies

Murine FGF21 (mFGF21) was prepared in our own lab as described previously [10]. Blood glucose test strips were purchased from Ningbo Cicheng Biochemical Reagent Company (Ningbo, China). Anti-SIRT1, anti-AMPKα, and anti-phospho-AMPKα ($pThr^{172}$) antibodies were purchased from Sigma (St Louis, USA), and TRIzol reagents were purchased from Invitrogen Company (Carlsbad, USA). All other reagents used in these experiments were obtained from the commercial source.

Mouse model of chronic ethanol gavage

All animals used in this study received humane care in compliance with the regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NIH Publication, 1996 edition. All protocols were approved by the Institutional Animal Care Committee of Northeast Agricultural University, China.

Eight to 10-week-old male KM mice (Yisi, Changchun, China) were fed with a nutritionally adequate liquid control diet (Bioserv, Frenchtown, USA) for 5 days and then were gavaged a single dose of ethanol (5 g/kg body weight, 40% ethanol) [11]. The gavage was always performed in the early morning. After gavage, mice were slow-moving but conscious, and regained normal behavior within 4–6 h. All animals were maintained in an air-conditioned room under controlled illumination (12 h light/dark cycle), temperature (22–24°C) and humidity (40%–60%), and had free access to standard rodent chow and tap water throughout the experimental period.

Treatment of mice with recombinant FGF21 protein

Mice were gavaged a single dose of ethanol (5 g/kg body weight, 40% ethanol) for 6 weeks and then further divided into two different groups: (i) standard chow treatment with equivalent volume of saline administered i.p.; and (ii) standard chow treatment with FGF21 (2 mg/kg/day dissolved in saline) administered i.p. After 3 weeks of treatment, mice were sacrificed after overnight fasting and serum was collected from the retro-orbital sinus of each non-anesthetized mouse.

Mouse serum assays

The whole blood sample was then centrifuged at 2500 *g* for 10 min to yield the serum fraction, which was used for subsequent biochemical analyses. TG, cholesterol, aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), and alkaline phosphatase (ALP) levels were determined by a DX800 programmed automatic biochemical analyzer (Beckman, Pasadena, USA) according to the manufacturer's specifications [8].

SOD, MDA, and GSH assays

Malondialdehyde (MDA) was quantified according to the thiobarbituric acid reaction as previously described [12,13] using a thiobarbituric acid reactive substances assay kit (Cayman Chemical Co., Inc., Ann Arbor, USA). In brief, 25 mg of liver tissue was added to 250 μl of radioimmuno precipitation assay buffer containing protease inhibitors. The mixture was sonicated for 15 s at 40 V over ice and centrifuged at 1600 *g* for 10 min at 4°C. The supernatant was used for analysis. SOD and GSH-PX were quantified with a commercially available kit (Jiancheng, Nanjing, China) and normalized by protein content.

Quantitative PCR analysis

Total RNAs were extracted from frozen mouse liver using TRIzol reagent. Then the RNA (0.5 μg) was reverse transcribed and subsequently amplified by TP800 Thermal Cycler Dice™ Real Time PCR (TaKaRa, Dalian, China). All quantitative PCRs were performed at least in triplicate. PCR values are corrected for the efficiency of the primer pairs and normalized to β-actin. The following ΔΔCT method was applied in the data analysis.

$$\text{Relative mRNA} = 2^{-[(CT_{\text{treated}} - CT_{\beta-\text{actin}}) - (CT_{\text{control}} - CT_{\beta-\text{actin}})]}$$

The following primer sequences targeting fatty acid synthase (FAS), acetyl-CoA-carboxylase (ACC1), and acetyl2-CoA-carboxylase (ACC2) were used for quantitative PCR analysis: *FAS* forward, 5′-CTCCTGCTGATGTGCTTCAT-3′; *FAS* reverse, 5′-TGAGCTGGGTGGGTAGGA-3′; *ACC1* forward, 5′-AGCAGATCCGCAGCTTG-3′; *ACC1* reverse, 5′-ACCT CTGCTCGCTGAGTGC-3′; *ACC2* forward, 5′-TTGGCCA AGCTATTGCGACA-3′; *ACC2* reverse, 5′-GCAAAGGCA TTGGCTGGAAG-3′; *β-actin* forward, 5′-GAGCGGGAA

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ATCGTGCGTAC-3′; and *β-actin* reverse, 5′-GCCTAGAA GCATTTGCGGTGGAC-3′.

Histopathological examination

Liver samples from each group were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. Formalin-fixed, paraffin-embedded sections were cut (5 μm thick) and mounted on glass slides. The sections were deparaffinized in xylene and stained with hematoxylin/eosin (HE) (Jiancheng) [14]. Finally, samples were analyzed microscopically with an IX51 microscope (Olympus, Shenzhen, China).

Culture of HepG2 cells

HepG2 human hepatoma cells were obtained from CTCC (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, USA) supplemented with 10% fetal bovine serum (Life Technologies). Alcohol-induced fatty liver model was achieved by adding ethanol (75 mM) into the medium and HepG2 cells were pretreated with FGF21 (5 nM) for 60 min prior to ethanol [15]. Every 24 h, media were replaced in both control and ethanol groups without or with FGF21 treatment with fresh media, respectively. Four days after seeding, cells were trypsinized and seeded into new flasks at two million viable cells per flask, with media changes continuing daily as before. Five days after the split (a total of 9 days without or with ethanol), cells were collected by trypsinization.

Detection of intracellular reactive oxygen species

To measure reactive oxygen species (ROS) in HepG2 cells produced by alcohol treatment, we used a DCFH-DA assay. DCFH-DA (Beyotime, Beijing, China) is a fluorescent dye that crosses the cell membrane and is enzymatically hydrolyzed by intracellular esterase to non-fluorescent DCFH. Cells were treated as described previously [15]. Cells were then incubated with DCFH-DA at a final concentration of 10 μM in high-glucose DMEM without FBS for 20 min at 37°C, washed three times with DMEM and analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, USA). For each analysis, 10,000 events were recorded [16].

Nile red staining and determination of intracellular lipid accumulation

To visualize the intracellular lipid droplets, cells cultured on glass coverslips were stained with Nile red (Jiancheng) for 20 min at 37°C [17]. Images were acquired with the IX51 microscope. The intracellular TG content was quantified with a commercially available kit (Jiancheng) and normalized by protein content.

Western blot analysis

Equal amounts of protein from liver lysates and HepG2 cells were resolved on SDS−PAGE [18] and trans-blotted onto NC membrane (Bio-Rad, Hercules, USA). A standard immunodetection protocol was employed using primary antibodies against SIRT1, AMPK, p-AMPK and β-actin (Sigma). Samples were detected using HyGloChemiluminescent HRP anti-rabbit antibodies (Beyotime) Detection Reagent on the Automated Bio-spectrum Imaging System (Sage Creation, Beijing, China).

Statistical analysis

Data were expressed as mean ± SEM of at least three separate experiments. Statistical analysis was performed by one-way analysis of variance and least significant density *post hoc* test with SPSS 20.0 (SPSS, Inc., Chicago, USA). $^*P < 0.05$ was considered to show statistically significant difference.

Results

Effect of FGF21 on body composition and food intake of alcohol-treated mice

The alcohol group showed an apparent reduction in the body weight, which was significantly decreased to 26.5 ± 1.36 g compared with the control group (33.1 ± 1.08 g) and the body weight was apparently reversed by FGF21 treatment (31.4 ± 1.01 g) compared with the alcohol group (**Fig. 1A**). The alcohol group showed a slow and progressive although not significant reduction in mean food intake, decreasing to 3.96 ± 0.9 g/day compared with the control group (4.55 ± 1.3 g/day); and the appetite was apparently reversed by FGF21 treatment (4.73 ± 0.9 g/day) compared with the alcohol group (**Fig. 1B**). The hepatic index (HI) was significantly higher in the alcohol group (2.87% ± 0.16%) than in the control group (2.63% ± 0.19%), despite the decreases in appetite and body weight in the alcohol group compared with the control group. The HI was reversed to the control level by the FGF21 treatment (2.68% ± 0.19%), indicating that FGF21 can significantly lessen the swelling of liver (**Fig. 1C**). These results suggest that FGF21 improves body composition and prevents hepatomegaly in the alcohol-treated mice, and thus ameliorates AFLD.

Effect of FGF21 on the liver functions

The levels of aminotransferases (ALT, AST, and ALP) and TBIL in serum were dramatically elevated in the alcohol group, indicating that the liver functions of alcohol-treated mice were severely damaged (**Table 1**). After 3 weeks of treatment with FGF21, the levels of serum biomarkers (ALT, ALP, and TBIL) reflecting liver functions were significantly lower than those of the alcohol group (**Table 1**), and the serum level of AST in the FGF21 group also showed a slight

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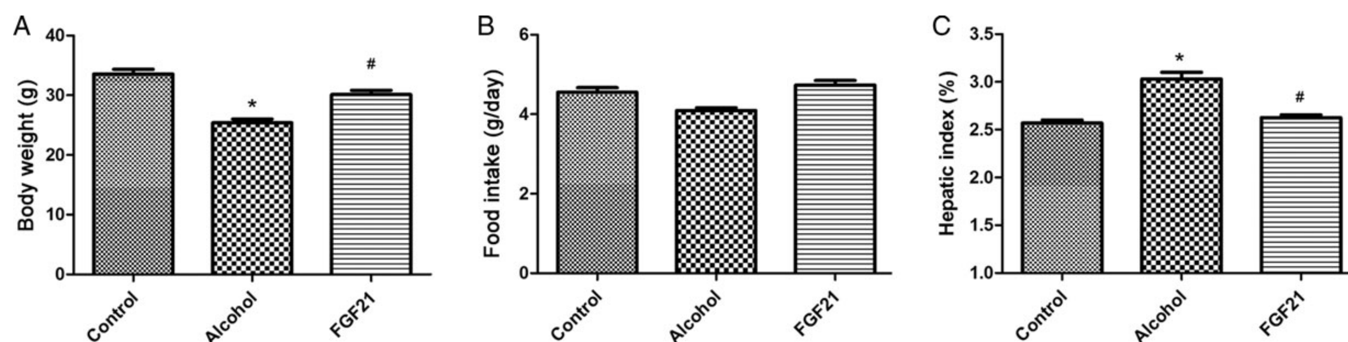

Figure 1. Effect of FGF21 on body composition and food intake of alcohol-treated mice (A) Effect of FGF21 on body weight. (B) Effect of FGF21 on daily food intake. (C) Effect of FGF21 on HI is calculated through liver weight/body weight. The values (mean ± SEM) shown are the average of eight independent measurements. $^{*}P<0.05$ vs. control group; $^{\#}P<0.05$ vs. alcohol group.

Table 1. Effect of FGF21 on the liver functions

Group	AST (U/L)	ALP (U/L)	ALT (U/L)	TBIL (U/L)
Control	241.25 ± 16.78	60.33 ± 7.60	63.31 ± 3.75	6.22 ± 1.25
Alcohol	316.67 ± 24.04*	80.33 ± 8.25*	100.21 ± 13.86**	12.31 ± 0.32**
FGF21	287.33 ± 5.24	58.42 ± 5.49#	69.25 ± 7.26##	9.38 ± 0.16##

Data were mean ± SEM of eight independent measurements.
$^{*}P<0.05$ and $^{**}P<0.01$ vs. control group; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. alcohol group.

Table 2. Effect of FGF21 on the changes in serum lipid parameters

Group	FBG (mM)	TG (mM)	TC (mM)	LDL-c (mM)	HDL-c (mM)
Control	5.03 ± 0.24	0.77 ± 0.22	2.87 ± 0.22	0.28 ± 0.11	1.76 ± 0.09
Alcohol	5.33 ± 0.52	1.07 ± 0.07**	3.71 ± 0.27*	0.46 ± 0.12**	1.85 ± 0.22
FGF21	4.97 ± 0.29	0.75 ± 0.17##	2.73 ± 0.21#	0.28 ± 0.03##	1.76 ± 0.37

Data were mean ± SEM of eight independent measurements.
$^{*}P<0.05$ and $^{**}P<0.01$ vs. control group; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. alcohol group.

decrease although not significant compared with that of alcohol group. Therefore, from the results above, we conclude that FGF21 improves liver functions.

Effect of FGF21 on the changes in serum lipid parameters

As expected, alcohol feeding significantly increased the levels of TG, TC, and LDL-c in serum to 1.07 ± 0.07, 3.71 ± 0.27, and 0.46 ± 0.01 mM, respectively. However, the HDL-c and FBG levels showed a slight increase but not significant compared with normal mice. FGF21 significantly lowered the level of TG (29.1% reduction compared with the alcohol model group), TC (26.4% reduction), and LDL-c (39.1% reduction) in serum (**Table 2**). These results indicate that FGF21 remarkably improves lipid metabolism.

Effect of FGF21 on oxidative stress

Chronic alcohol administration induced significant oxidative stress, as evidenced by increased hepatic MDA (4.43 ± 0.52 nmol/mg) and GSH-PX (123.71 ± 14.27 U/mg) levels in the alcohol group. FGF21 effectively reduced the hepatic MDA and GSH-PX levels to 3.27 ± 0.49 nmol/mg and 112.73 ± 10.21 U/mg respectively compared with the alcohol group. Moreover, the level of SOD was reduced in some degree in the alcohol group (32.47 ± 1.07 U/mg), and FGF21 treatment markedly increased the SOD level, which was similar to that of the control group (**Table 3**).

Table 3. Effect of FGF21 on oxidative stress

Group	MDA (nM/mg)	SOD (U/mg)	GSH-PX (U/mg)
Control	3.03 ± 0.24	37.77 ± 3.42	104.87 ± 4.22
Alcohol	4.43 ± 0.52**	30.17 ± 1.07*	123.71 ± 2.17*
FGF21	3.27 ± 0.49##	38.75 ± 2.47#	112.73 ± 3.72#

Data were mean ± SEM of eight independent measurements.
$^{*}P<0.05$ and $^{**}P<0.01$ vs. control group; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. alcohol group.

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Effect of FGF21 on the expression of lipid metabolism-related genes

As lipid parameters were dramatically increased in alcohol-treated mice, we also determined the relative mRNA expression of FAS, ACC1, and ACC2 (**Fig. 2**), which were key genes related to fat synthesis in liver tissues. Alcohol-treated mice showed significantly higher level of mRNA expression of FAS (1 ± 0.11), ACC1 (1 ± 0.13), and ACC2 (1 ± 0.14) compared with control mice. FGF21 administration markedly suppressed the mRNA expression of FAS (0.14 ± 0.01), ACC1 (0.56 ± 0.08), and ACC2 (0.53 ± 0.06) to the normal level.

Effect of FGF21 on the liver morphology

Histological sections of the liver tissues from the alcohol-treated mice reflected the strikingly pathologic changes of AFLD compared with those of the normal control littermates (**Fig. 3**). Under the light microscope, the livers of the alcohol-treated group showed severe swelling and a large number of small vacuoles in the liver cytoplasm especially the Chuo of hepatic lobule. Little fat vacuoles were visible within the cytoplasm of hepatocytes in the FGF21-treated-mice, but the amounts were significantly less than that of alcohol-treated alone, indicating that the FGF21-treated mice almost restored the liver morphology to normal, which further corroborated that FGF21 has the potential to become a powerful therapy to treat AFLD.

FGF21 attenuates intracellular ROS production induced by alcohol

To measure intracellular ROS production in HepG2 cells after alcohol treatment and the effect of FGF21 on ROS production, we used a DCFH-DA assay. As shown in **Fig. 4**, the ROS production of the alcohol group was increased by 2159.6% compared with that of the control group. Pretreatment with FGF21 (5 nM) resulted in intracellular ROS production decreasing by 70.4% compared with that of the alcohol group. These results indicated that FGF21 treatment significantly suppressed

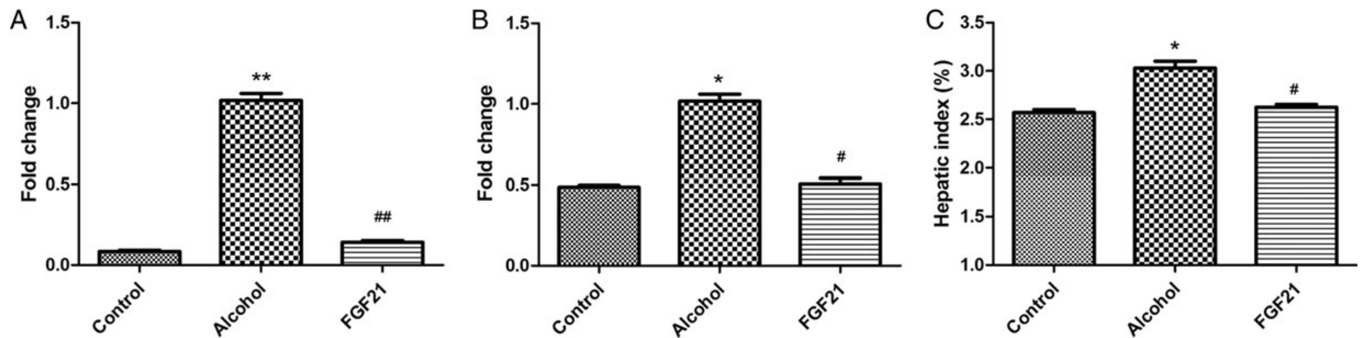

Figure 2. Effect of FGF21 on changes in gene expression related to lipid metabolism Real-time PCR analysis of FAS, ACC1, and ACC2 mRNA expression in liver, all were performed at the end of the study after overnight fasting. Levels of FAS, ACC1, and ACC2 transcripts were assessed by real-time PCR using SYBR green in liver samples from five mice from each treatment group at the end of the study. The values (mean ± SEM) shown are the average of eight independent measurements. $^{*}P<0.05$ and $^{**}P<0.01$ vs. control group; $^{\#}P<0.05$ vs. alcohol group.

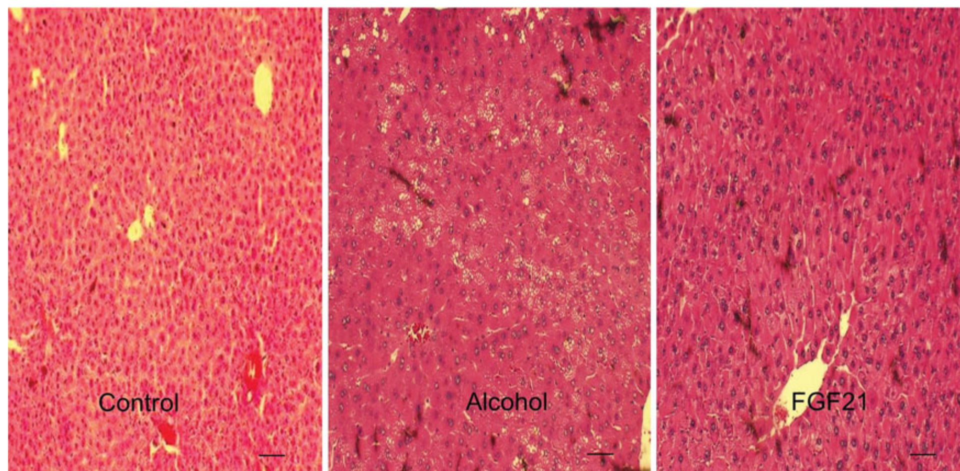

Figure 3. Effect of FGF21 on the liver morphology Samples of liver were resected and fixed in 10% formaldehyde phosphate buffer saline (PBS, pH 7.4), then embedded in paraffin, sectioned, stained with HE. Finally were analyzed microscopically. Scale bar: 1 μm.

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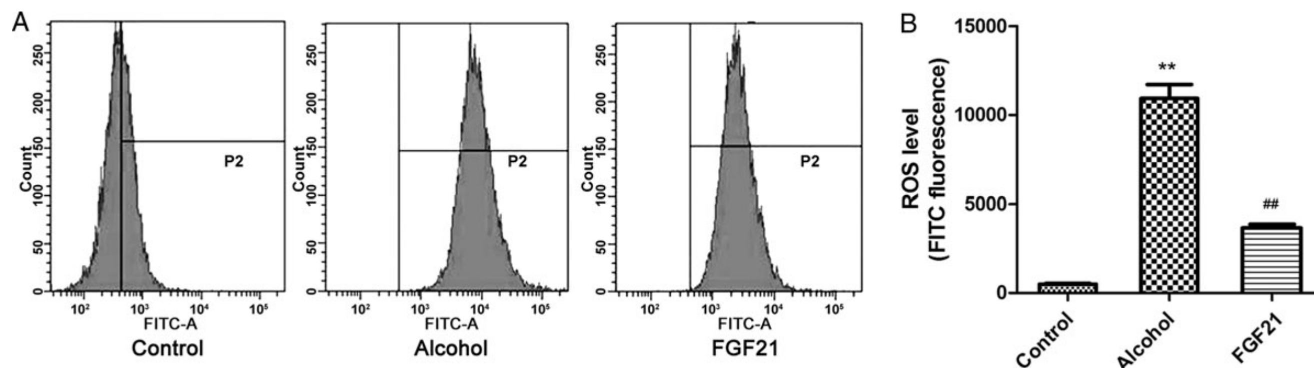

Figure 4. FGF21 attenuates intracellular ROS production after alcohol-induced oxidative stress HepG2 cells were treated as indicated by the various groups. Intracellular ROS production was determined by DCFH-DA fluorescence. $^{**}P<0.01$ vs. control group; $^{\#\#}P<0.01$ vs. alcohol group.

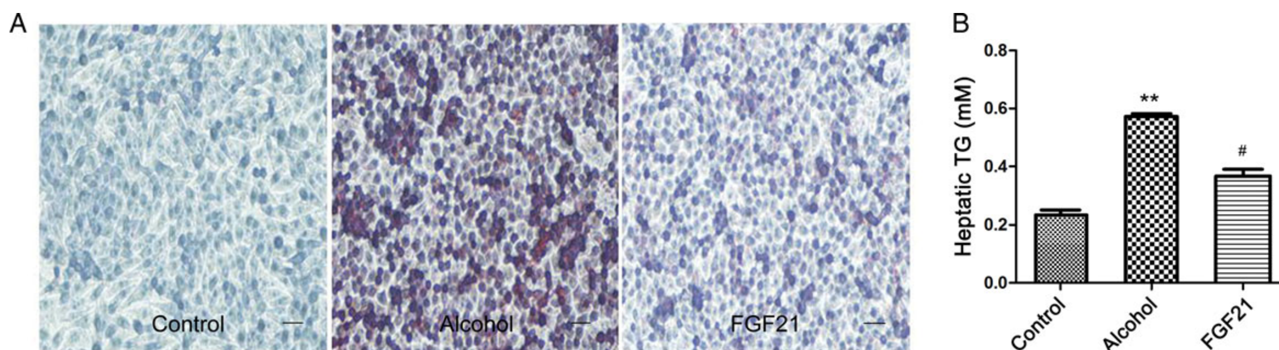

Figure 5. FGF21 treatment reduces the fatty acid load in hepatocytes Cells cultured on glass coverslips were stained with Nile red for 20 min at 37°C. Images were acquired with the microscope. Scale bar: 1 μm. The intracellular TG content was quantified with a commercially available kit and normalized by protein content. $^{**}P<0.01$ vs. control group; $^{\#}P<0.05$ vs. alcohol group.

intracellular ROS production induced by alcohol in HepG2 cells. Therefore, FGF21 showed a significant protective effect against alcohol-induced oxidative stress.

Nile red staining and determination of intracellular lipid accumulation

We employed HepG2 cells as the *in vitro* model to study AFLD. Fat loading was observed by staining the lipid droplets with Nile red. Microscopic observations of alcohol-treated HepG2 cells revealed that conspicuous steatosis (**Fig. 5A**) was caused by the saturated fatty acids. We treated HepG2 cells engorged with lipid droplets with FGF21 to determine whether it led to a reduction in fat store. We observed that steatosis, lipid-droplet number, and size were significantly reduced after FGF21 treatment (**Fig. 5A**). Quantification of TG corroborated these findings and demonstrated a 44.89% reduction in TG level compared with alcohol-treated alone (**Fig. 5B**).

FGF21 activates the AMPK- SIRT1 pathway

To explore whether FGF21 increases AMPK activity *in vivo* and *in vitro*, we administered recombinant FGF21 protein to alcohol-treated mice for 3 weeks and the levels of

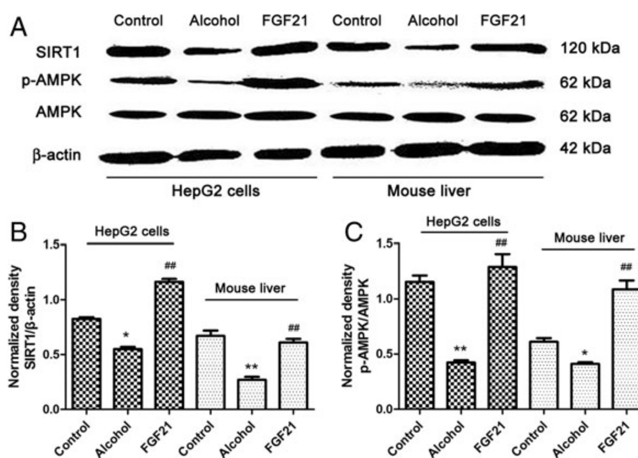

Figure 6. FGF21 activates the AMPK-SIRT1 pathway (A) Western blot of liver lysates and HepG2 cells for SIRT1, AMPK, and p-AMPK. (B,C) Increased conversion of AMPK to p-AMPK (B) and increased SIRT1 (C) in samples treated with FGF21. $^{*}P<0.05$ and $^{**}P<0.01$ vs. control group; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. alcohol group.

phosphorylated AMPK in different groups were analyzed. As shown in **Fig. 6**, a significant decline was found in the level of p-AMPK in alcohol-treated alone, while the levels of p-AMPK increased by 154.9% in FGF21-treated mice, indicating enhanced activation of AMPK. Moreover, FGF21

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showed the similar effect on the level of phosphorylated AMPK in the HepG2 cell model (178% increase). These data suggest that FGF21 regulates the energy expenditure through activation of AMPK. SIRT1 protein expression level was markedly increased (119.1% and 110.9% increase, respectively, $P < 0.01$) *in vivo* and *in vitro* after treatment with FGF21, compared with that of alcohol treatment alone.

Discussion

Clinically, AFLD is characterized by increased accumulation of fat in the livers of patients who have consumed excessive amounts of alcohol for prolonged periods. Considerable evidence has shown that increased fat accumulation in liver can progress to more harmful forms of liver injury such as fibrosis and cirrhosis in humans [19]. Despite the profound damage impact of AFLD, little progress has been made in the management of patients with this severe clinical condition. No new drugs for AFLD have been successfully developed since the early 1970s, at which time the use of corticosteroids was proposed for the treatment of severe alcoholic hepatitis [20].

The molecular and cellular mechanisms by which ethanol causes AFLD are multiple and still incompletely understood. There is growing evidence to suggest that the SIRT1-AMPK signaling system is an essential regulator of hepatic fatty acid oxidation and is inhibited by chronic alcohol exposure. Furthermore, this pathway is closely associated with the pathogenesis of AFLD [21].

FGF21, a new member of the FGF family, is characterized as a novel metabolic regulator and predominantly expressed in murine liver and thymus [22]. Our previous study showed that exogenous FGF21 can inhibit lipid synthesis, reduce hepatic TG accumulation, enhance glucose metabolism, and inhibit gluconeogenesis in the liver, and can thus reverse hepatic steatosis and fibrosis and prevent NAFLD. Additionally, FGF21 regulates energy homeostasis in adipocytes through activation of AMPK and SIRT1, resulting in enhanced mitochondrial oxidative function [9]. Thus, FGF21 has the potential to become a powerful therapy to treat AFLD.

In the present study, we investigated the effects of FGF21 on the AFLD model *in vivo* and *in vitro*, and whether FGF21 has therapeutic effects on AFLD via activation of the AMPK-SIRT1 pathway. It was found that FGF21 had little effect on normal mice (data not shown). However, FGF21 had positive effects in the AFLD mice by improving body composition, ameliorating serum lipid profiles, suppressing hepatocyte lipid droplet accumulation and decreasing oxidative stress. FGF21 effectively reduced the hepatic MDA and GSH-PX levels (3.27 ± 0.49 nmol/mg and 112.73 ± 10.21 U/mg vs. the alcohol group) and markedly increased the SOD level to that in the control group (**Table 3**). These data suggested that FGF21 has a significant protective effect against alcohol-induced oxidative stress (**Fig. 3**) as SOD, a typical class of antioxidant enzymes, can remove ROS and reduce oxidative stress-induced damage [23] Furthermore, FGF21 was found to have marked effects against pathologic liver conditions, which were confirmed through histopathological examination (**Fig. 3)** and serum assay of biomarkers that reflect liver functions (**Table 1**). Therefore, we conclude that FGF21 corrects multiple metabolic parameters and significantly ameliorates AFLD in the murine model created by chronic ethanol gavage.

It is generally accepted that the AMPK-SIRT1 signaling system plays a vital role in the development of AFLD [21]. Alcohol consumption inhibits AMPK activity in the liver, leading to decreased phosphorylation, increased activity of ACC, and decreased activity of carnitine palmitoyltransferase 1, which each has an important role in the development of alcoholic fatty liver [4,5]. Thus, ethanol's effects on AMPK appear to play a role in both the decreased fatty acid oxidation and the increased fatty acid synthesis seen in alcoholic liver disease. Here, we showed that FGF21 therapy up-regulated SIRT1expression and stimulated AMPK activity in the livers of alcohol-fed mice. FGF21-mediated activation of the SIRT1-AMPK system was accompanied by decreased expression of lipogenic genes, such as FAS, ACC1, and ACC2 [24].

Taken together, we hypothesize that ethanol's effect on liver partially inhibits AMPK. Hence, ethanol-mediated dysregulation of the AMPK-SIRT1 signaling pathway contributes to the development of AFLD. Administration of FGF21 significantly stimulated AMPK activity and thus reversed lipid metabolic disorders.

Increased generation of ROS occurs in AFLD and this has long been considered to play an important role in progression to steatohepatitis [25]. When ROS production exceeds the buffering/detoxifying capacity of antioxidant systems, various cellular macromolecules are subject to direct oxidative attack [26]. Therefore, ROS production is responsible for liver damage during AFLD. To address the role of FGF21 in AFLD in human liver, we studied the well-characterized HepG2 cell line, which retains many liver-specific metabolic functions. Our results showed that FGF21 treatment significantly suppressed intracellular ROS production in HepG2 cells. As expected, the results of western blot once again demonstrated that FGF21 significantly increased SIRT1 expression and AMPK activity. As a consequence, FGF21 reduced fat accumulation in HepG2 cell model that had abnormal lipid metabolism and fat storage (**Fig. 5A**). Hence, we deduced that the protective effects of FGF21 against alcohol-induced liver steatosis may be realized, at least in part, by turning on the hepatic SIRT1-AMPK signaling system to ameliorate alcohol-induced impairments in the signaling pathways controlling lipid metabolism.

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In summary, the present study suggests that FGF21 administration can ameliorate AFLD by activating the SIRT1-AMPK pathway. These findings raise the possibility that FGF21 may be a promising therapy in the treatment or prevention of AFLD and its associated complications. To the best of our knowledge, this is the first study to show the therapeutic effect of FGF21 in alcoholic fatty liver.

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