DOI: 10.26599/FSHW.2025.9250849

Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Isocaloric high protein dietary pattern ameliorates chronic alcohol-induced liver dysfunction *via* a BDH2 involved mechanism

Jiannan Qiu [1, 2, #], Fan Dong [1, #], Kaixin Pan [1, #], Tianchi Sun [1], Feiwei Cao [1], Qinchao Ding [1], Qianyu Qian [2], Xiaobing Dou [2], Qing Song [1, *], Songtao Li [1, *]

[1] *School of Public Health, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, PR. China*

[2] *School of Life Sciences, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, PR. China*

ABSTRACT: Emerging evidence shows that the proportion of dietary macronutrients determines the pathological progression of multiple metabolic diseases, however, whether and how this affect alcohol-associated liver diseases (ALD) are largely unclear. The present study was conducted to explore the role of dietary protein proportions on ALD and to unveil its potential mechanisms. C57BL/6N mice were administrated with isocaloric Lieber-DeCarli alcohol liquid diets with different proportions of protein, including normal protein (NP, **15% energy**), high protein (HP, 30% energy), and low protein (LP, 10% energy), respectively. Our results showed that HP diet significantly ameliorated alcohol-induced hepatic steatosis, liver injury, oxidative stress, and inflammation, which were aggravated in LP diet-fed mice. Alcohol consumption-stimulated lipid metabolism-associated genes dysregulation was improved by HP diet. Liver transcriptome sequencing analysis and further validation identified that alcohol dramatically down-regulated 3-hydroxybutyrate dehydrogenase 2 (*Bdh2*) along with the reduction of β-hydroxybutyric acid, which were rescued by HP diet intervention. *Bdh2* was also decreased in the liver of patients with ALD and alcohol-exposed hepatocytes. Spearman correlation analysis revealed that *Bdh2* was negatively associated with the indices of hepatic steatosis, liver injury, and oxidative stress. Genetically knocking-down *Bdh2* significantly enhanced alcohol-induced hepatotoxicity, lipid accumulation, and pro-inflammatory factors expression in cultured hepatocytes. In summary, HP diet intervention alleviates chronic alcohol consumption induced liver dysfunction in mice. BDH2 is a potential novel target for ALD prevention *via* restoring the ketogenic pathway that contributes to HP diet-improved ALD. HP dietary pattern might be a promising choice for ALD management.

Key words: high-protein diet, alcohol-associated liver disease, Bdh2, hepatic steatosis, liver injury

1. Introduction

Alcohol-associated liver disease (ALD) is predominantly characterized by the pathological accumulation of fat and inflammatory damage within the liver, frequently resulting from chronic and excess alcohol consumption[1, 2]. This progressive disorder may advance from initial stages of steatosis and inflammation to more severe hepatic conditions, such as alcoholic hepatitis, cirrhosis, and potentially hepatocellular carcinoma, if not appropriately managed[3, 4]. Patients with ALD are usually suffered to poor quality of life and life expectancy, while also placing a considerable economic burden on public health systems[5, 6].

# These authors contribute equally to this paper.
***Corresponding author**
lisongtao@zcmu.edu.cn; songqingbetty@163.com

Received 24 April 2025
Received in revised from 25 May 2025
Accepted 12 August 2025

Consequently, comprehensive understanding the pathophysiology of ALD and developing effective treatment strategies are critical priorities in contemporary medical research.

In the past several decades, many efforts have been made to disclose the pathological basis the of ALD. In liver, ethanol is metabolized by alcohol dehydrogenase (ADH), microsomal ethanol oxidizing system (MEOS, mainly by cytochrome P450 2E1, CYP2E1), and catalase, to produce acetaldehyde, which is further oxidized by aldehyde dehydrogenase (ALDH) to generate acetic acid[7]. Long term excessive alcohol consumption stimulates CYP2E1 upregulation and activation, which leads to excessive reactive oxygen species (ROS) generation during ethanol oxygenolysis, and further induces oxidative stress, resulting in the dysregulation of lipid metabolism related genes and subsequent hepatic lipid accumulation[8, 9]. Excessive lipids, especially non-esterified fatty acids, induce lipotoxicity and liver injury[10]. Oxidative stress, lipotoxicity, acetaldehyde, and other stimuli together promote the activation of Kupffer cells and the infiltration of neutrophils and monocytes from circulating, accelerating hepatitis progress[11, 12].

Nutritional status directly affects the quality of life and survival period of patients with ALD[13]. Nutritional therapy has been recognized as an essential strategy in mitigating the pathological process of ALD, since approximately 50% of outpatients and nearly all inpatients with ALD exhibit varying degrees of malnutrition[13]. Emerging evidence showed that reasonable modification of dietary pattern, especially for the proportion of dietary macronutrients, can help improve various metabolic diseases, such as obesity, type 2 diabetes, and non-alcoholic fatty liver disease[14, 15]. Recently, we reported that isocaloric low carbohydrate dietary pattern ameliorates chronic alcohol consumption-induced liver dysfunction in mice, indicating dietary patterns modification might be a promising choice for ALD management[16]. Patients with ALD are often accompanied by protein malnutrition[17]. Accumulating evidence showed the beneficial role of increasing the dietary proportion of protein on the improvement of intrahepatic lipid deposition in the patients with metabolic disorders[15, 18-22]. However, whether and how dietary protein ratio affect ALD are still largely unclear. The present study was conducted to demonstrate the regulation of different proportion of dietary protein on ALD, and elucidate its potential mechanisms.

2. Materials and methods

2.1 Animal experiments

The animal experiment was approved by the Animal Experimental Ethics Committee of Zhejiang Chinese Medical University, with the approval number ZSLL-2018-008. Eight-week-old male C57BL/6N mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd, Shanghai, China (License No. SCXK (Hu) 2017-0005). All mice were housed under controlled environmental conditions, maintained at a temperature of (23 ± 2)°C, relative humidity of (55 ± 5)%, and a 12 h light/dark cycle. After one week acclimatization, the mice were provided with a modified Lieber-DeCarli liquid diet, manufactured by Fubo Biotechnology (Beijing, China). The mice were randomly divided into four groups (n = 8/group): a) pair-feed (PF) group, b) alcohol-feed (AF) group, c) high-protein (HP) + AF group, and d) low-protein (LP) + AF

group. The feed was freshly prepared daily, with the specific compositions detailed in Table S1. To ensure that equal volumes of the feed contained equivalent energy content, the energy derived from alcohol was compensated by reducing the quantities of macronutrients, while maintaining a consistent macronutrient ratio across all dietary groups. A constant proportion of fat was maintained in this study, and the alteration of protein ratio was compensated by the regulating of carbohydrates. Each group of animals received an equal amount of diet every day. The diet amount was given according to the recorded minimum food intake of the cage on the previous day. During the initial three days, all mice were provided with a modified Lieber-DeCarli liquid diet devoid of alcohol to facilitate adaptation to the liquid diet. Subsequently, the alcohol-fed groups received a Lieber-DeCarli liquid diet with a gradually increased ethanol concentration: 0.99% (v/v) on days 4-5, 1.99% on days 6-7, 3.98% during the second week, 4.88% during the third week, and 5.79% from the 4^{th} to the 8^{th} week. Food intake and the body weight of mice in each group were recorded daily and weekly, respectively. At the end of the experiment, the mice were subjected to a single binge ethanol exposure (4 g/kg body weight, 33%, v/v). After 6 h, all mice were euthanized using a pentobarbital solution (30 mg/kg body weight, ip). Plasma and liver samples were collected for further analysis.

2.2 Biochemical analysis

Commercial assay kits, including alanine transaminase (ALT) microplate test kit (C010-2), aspartate transaminase (AST) microplate test kit (C009-2), triglycerides (TG) assay kit (A110-1-1), total cholesterol (TC) assay kit (A111-1-1), low-density lipoprotein cholesterol (LDL-c) assay kit (A113-1-1), and high-density lipoprotein cholesterol (HDL-c) assay kit (A112-1-1), were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). The levels of malondialdehyde (MDA, S0131S), superoxide dismutase (SOD, S0101S), glutathione peroxidase (GSH-Px, S0056), and glutathione/oxidized glutathione (GSH/GSSG, S0053) in the liver were detected by commercial assay kits, which were obtained from Beyotime Biotechnology Research Institute (Shanghai, China). Bodipy 493/503 (C2053S) and DCFH-DA (S0034S) staining kits were also purchased from Beyotime Biotechnology Research Institute (Shanghai, China). The contents of hepatic β-hydroxybutyrate dehydrogenase 2 (BDH2, JM-13516M1) and β-hydroxybutyric acid (β-HB, BC5085) were detected by commercial assay kits purchased from JingMei Biotechnology (Jiangsu, China) and Solarbio Science & Technology Co., Ltd (Beijing, China), respectively.

2.3 Histological examination

The portion of the liver samples were fixed in 4% paraformaldehyde, subsequently transferred to a 30% sucrose solution for osmotic dehydration, then embedded in an optimal cutting temperature (OCT) embedding agent. Following rapid freezing in liquid nitrogen, the embedded tissue was sectioned, and the sections were adhered to 8 μm-thick slides prior to Oil Red O staining. Additionally, another portion of the liver was dehydrated, embedded in paraffin, sliced, and finally stained with hematoxylin and eosin (H&E) solution. The Oil Red O and H&E staining solutions were obtained from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). The positively stained areas were used to qualified lipid accumulation using Image J software (Fiji version 1.8.0)

2.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from liver tissues utilizing the TRIzol® reagent (Invitrogen, Carlsbad, CA) according to standardized protocols. The extracted RNA was then transcribed into complementary DNA (cDNA) following the rigorous guidelines provided by the reverse transcription kit (Promega, Madison, WI). The cDNA was subsequently amplified in a 384-well PCR plate using the SYBR Green/ROX qPCR premix (2×) on the QuantStudio™ Pro real-time PCR system (Thermo Fisher Scientific), ensuring precision and reproducibility. Gene expression levels were normalized to *18S* rRNA and were quantitatively analyzed using the $2^{-\Delta\Delta Ct}$ method. All primers employed in this study were custom-synthesized by Tsingke Biotechnology Co. Ltd. (Beijing, China), with specific sequences were detailed in Supplementary Table 2, facilitating reproducibility and validation of the experimental findings.

2.5 Western-blot analysis

As previously detailed, the Western blot was performed[23]. Protein concentrations in tissues were quantified using the BCA protein assay reagent. After antibodies incubation, including anti-CYP2E1 (19937-1-AP, Proteintech, USA), anti-ADH1 (sc-137078, Santa Cruz Biotechnology, USA), and anti-GAPDH (60004-1-Ig, Proteintech, USA), the chemiluminescence detection kit (Vazyme, Nanjing, China) was used to observe the immunoreactivity of protein expression. Band densities were quantified using Image-J software for quantitative analysis.

2.6 The transcriptome sequencing analysis

In accordance with the manufacturer's specifications, total RNA was extracted from liver tissues utilizing the TRIzol® reagent sourced from Invitrogen. Subsequent rigorous validation ensured that the extracted RNA met the stringent criteria for integrity and purity, thereby affirming its suitability for downstream applications. Subsequently, the processed total RNA was entrusted to Shanghai Majorbio Bio-Pharm Technology for the preparation of a high-quality sequencing library and subsequent sequencing on the advanced Illumina NovaSeq 6000 platform. Data derived from this RNA-Seq procedure underwent comprehensive analysis utilizing the Majorbio I-Sanger Cloud Platform (http://www.i-sanger.com), a reputable free online resource renowned for its robust analytical capabilities. To facilitate scientific reproducibility and data sharing, the resulting RNA-Seq datasets were deposited in the NCBI Sequence Read Archive (SRA) database, assigned with the unique accession number PRJNA1073686.

2.7 Cell culture

AML-12 mouse hepatocytes, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were cultured at 37°C in a controlled atmosphere consisting of 95% air and 5% CO_2. Cells were maintained in a customized DMEM/F12 medium with 10% (v/v) fetal bovine serum (FBS, Saiyamei Cell Technology Beijing Co., Ltd.), containing insulin (10 μg/mL), transferrin (5 μg/mL), selenium (5 ng/mL), and 40 ng/mL of dexamethasone (Sigma-Aldrich, St. Louis, MO).

2.8 Gene knockdown

Specific *si*RNAs (Santa Cruz Biotechnology, CA) for *BDH2* and chromatin remodeling protein microrchidia family CW-type zinc finger 4 (*MORC4*) were transfected into AML-12 hepatocytes using Lipofectamine 3000 (Thermo Fisher Scientific, MA), according to the manufacturer's protocol, respectively. Scrambled *si*RNA was used as control (Santa Cruz Biotechnology, CA).

2.9 Bodipy and ROS staining

AML-12 hepatocytes were incubated with Bodipy 493/503 dye (2 μmol/L in the culture medium) or DCFH-DA dye (10 μmol/L in the culture medium) at 37℃ for 30 min. Subsequently, images were acquired using a fluorescence microscope (ZEISS, Oberkochen, Germany), employing an optimally configured combination of filters to ensure precise visualization and quantification of the fluorescent signal. Using Image J (Fiji version 1.8.0), bodipy and ROS fluorescence quantifications were performed by measuring the mean fluorescence intensity after background subtraction, followed by normalization to the control samples to ensure accurate and reproducible analysis of lipid accumulation and reactive oxygen species.

2.10 Cell viability

AML12 hepatocytes were seeded in the plates in the previous day. Cells were treated at 50%-70% confluence. Cell viability was assessed after the procedure was finished using the Cell Counting Kit-8 (CCK8) assay (Beyotime, China) according to the manufacturer's instructions.

2.11 Statistical analysis

All data were analyzed using GraphPad Prism 8.0.1 software (GraphPad Software, CA) and were represented as mean ± standard deviation (SD). Two groups were compared by two-tailed Student *t*-tests. In the analysis of multi-group data, different statistical methods are employed based on the characteristics of the variables. For variables exhibiting a normal distribution with homogeneity of variances, a one-way ANOVA followed by the Least Significant Difference (LSD) test is utilized for inter-group comparisons. In cases where variables follow a normal distribution but display heterogeneity of variances, the Welch's ANOVA is applied for inter-group comparisons. For variables that do not conform to a normal distribution, the Kruskal-Wallis test, a non-parametric rank-sum method, is employed for inter-group comparisons. Comparisons between two groups are conducted using the *t*-test. $P < 0.05$ was considered to represent statistical significance.

3. Results

3.1 HP dietary pattern alleviates alcohol-induced liver injury and hepatic steatosis

As shown in Fig. 1A, compared to PF group, a significant reduction of body weight was observed in AF group from the 6th to the 8th week, which was further reduced in LP+AF group, while HP diet intervention markedly rescued chronic alcohol consumption-induced body weight loss. Food intake was recorded and showed in Fig. S1, mice from each group consumed equivalent energy from the diet. Elevated levels of plasma ALT and AST and liver morphological ballooning degeneration serve as hallmark indicators of hepatic damage, providing essential diagnostic insight for evaluating whether the ALD model was successfully established. In this study, chronic alcohol-fed significantly increased the liver weight and liver to body weight

ratio, as well as the activities of ALT and AST in the plasma (Fig. 1B-E). H&E staining further revealed that chronic alcohol consumption induced liver injury (Fig. 1F). Notably, the aforementioned pathophysiological alterations were markedly ameliorated by the HP diet intervention (Fig. 1B-F), whereas the LP diet enhanced alcohol-increased liver to body weight ratio and H&E analysis injury scores (Fig. 1C & F).

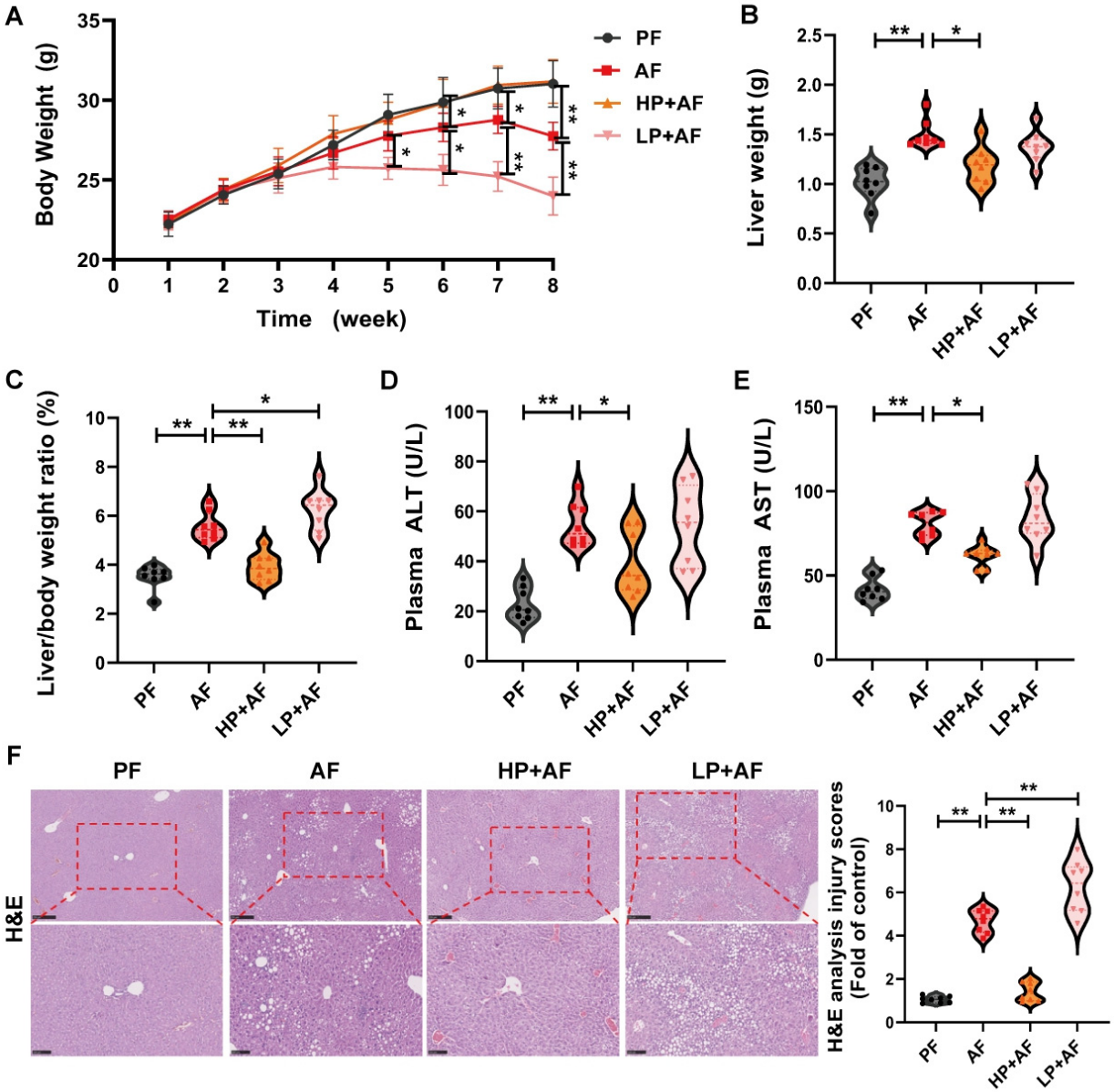


Figure 1. HP dietary pattern alleviates alcohol-induced liver injury. Male C57BL/6N mice (8-week-old) were pair-fed (PF), alcohol-fed (AF), high protein alcohol-fed (HP+AF) or low-protein alcohol-fed (LP+AF) for 8 weeks. (A) Dynamic changes in body weight. (B) Liver weight. (C) Liver to body weight ratio. (D) Plasma ALT. (E) Plasma AST. (F) Liver H&E staining (scale bar: 250 μm for the overall, and 100 μm for the detailed) and the H&E analysis injury scores. Data are presented as means ± SD (n = 8). *$P < 0.05$, and **$P < 0.01$ indicate statistically significant.

Hepatic steatosis is a classic pathological feature of ALD. Here, we observed that chronic alcohol intake significantly aggravated lipid accumulation in the liver, based on the measurements of Oil Red O stain and liver TG and TC contents (Fig. 2A-C). Alcohol-induced hepatic steatosis was obviously deteriorated by LP diet treatment, but reversed by HP diet intervention (Fig. 2A-C). Additionally, HP diet administration also rescued alcohol-induced dyslipidemia, which was manifested by the observations that alcohol-induced the

elevation of plasma TC, TG, and LDL-c, and the reduction of plasma HDL-c were significantly reversed (Fig. 2D-G), while LP diet treatment aggravated alcohol-induced dyslipidemia (Fig. 2D-G).

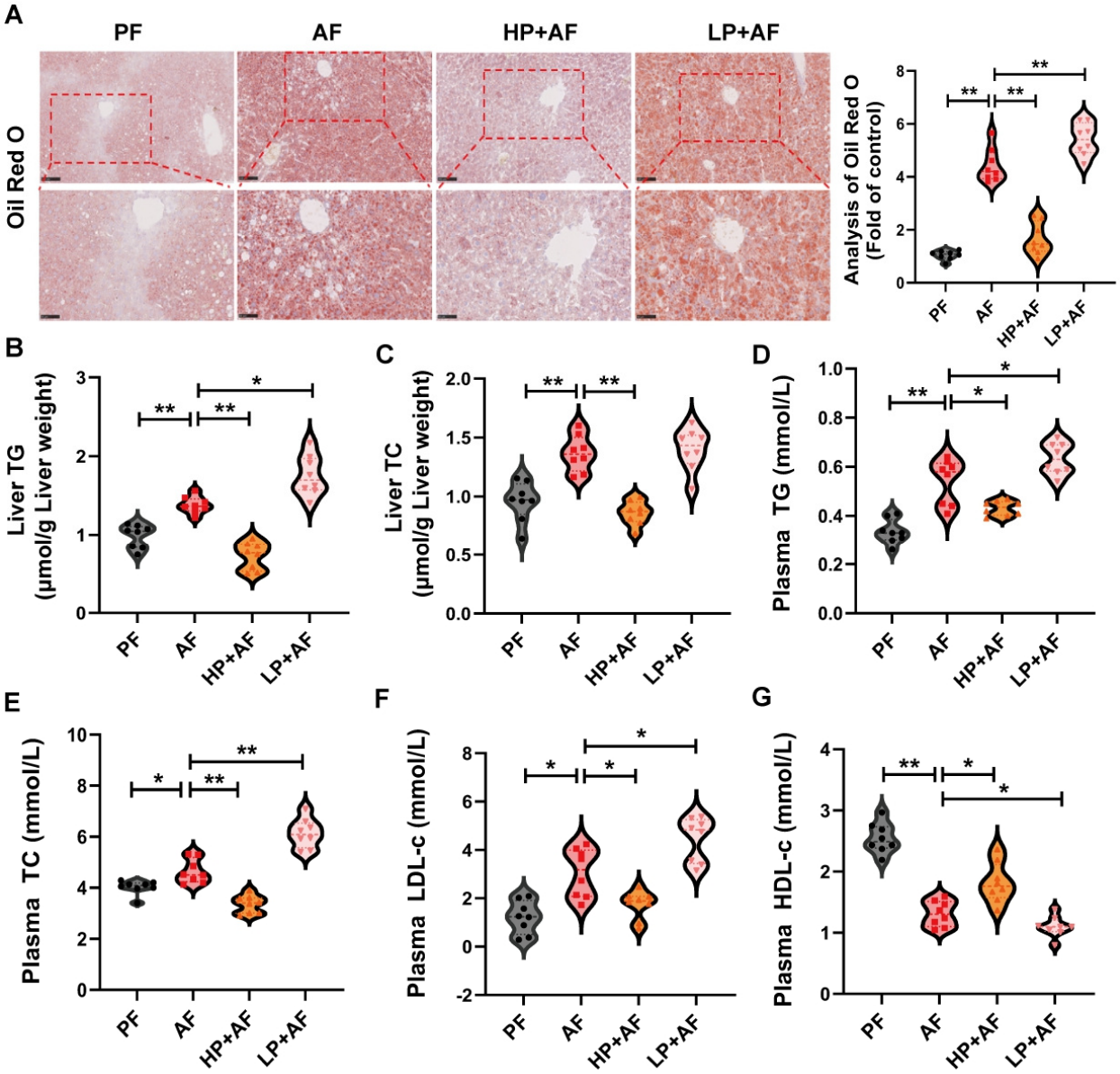
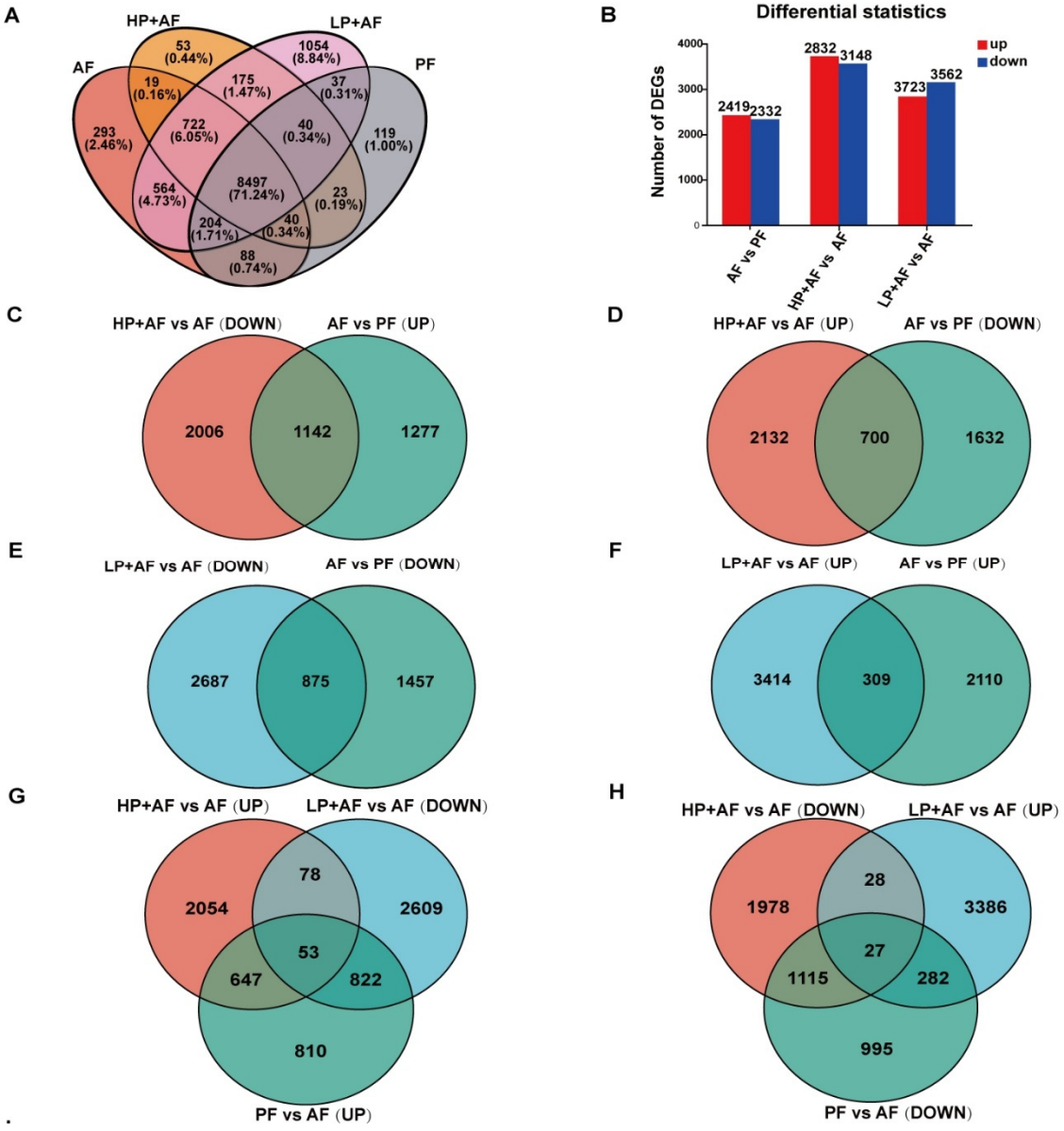


Figure 2. HP dietary pattern alleviates alcohol-induced hepatic steatosis. (A) Liver Oil red O stain (250 μm for the overall, and 100 μm for the detailed) and the analysis of positively stained areas. (B) Liver TG. (C) Liver TC. (D) Plasma TG. (E) Plasma TC. (F) Plasma LDL-c. (G) Plasma HDL-c. Data are presented as means ± SD (n = 8). *$P < 0.05$, and **$P < 0.01$ indicate statistically significant.

3.2 HP dietary pattern-protected ALD is associated with lipid metabolism, ROS, inflammation, and ketone pathways

To reveal the molecular mechanisms underlying the protective effects of HP diet against ALD, transcriptome sequencing analysis was conducted on the samples from liver. A total of 11928 genes were identified in the liver samples, with 8497 genes consistently detected across all the four experimental groups (Fig. 3A). Compared to PF group, a total of 2419 and 2332 genes were upregulated and downregulated in AF group, respectively (Fig. 3B). While, when compared to AF group, a total of 2832 and 3148 genes were upregulated and downregulated in HP+AF group, respectively (Fig. 3B), among which, 1142 alcohol-upregulated genes and 700 alcohol-downregulated genes were reversed by HP diet intervention (Fig.

3C & D). Meanwhile, when compared to AF group, a total of 3723 and 3562 genes were upregulated and downregulated in LP+AF group, respectively (Fig. 3B), among which, 309 alcohol-upregulated genes and 875 alcohol-downregulated genes were aggravated by LP diet intervention (Fig. 3E & F). After taking the intersection of all the data, a total of 27 alcohol-upregulated genes and 53 alcohol-downregulated genes were rescued by HP diet but were aggravated by LP diet (Fig.3G & H). Moreover, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted to elucidate the functional implications of the differentially expressed genes among the tested groups. Our data showed that the protective role of HP diet mainly associated with multiple pathways, including lipid metabolic process, ROS, inflammatory response, and response to ketone (Fig. 3I).



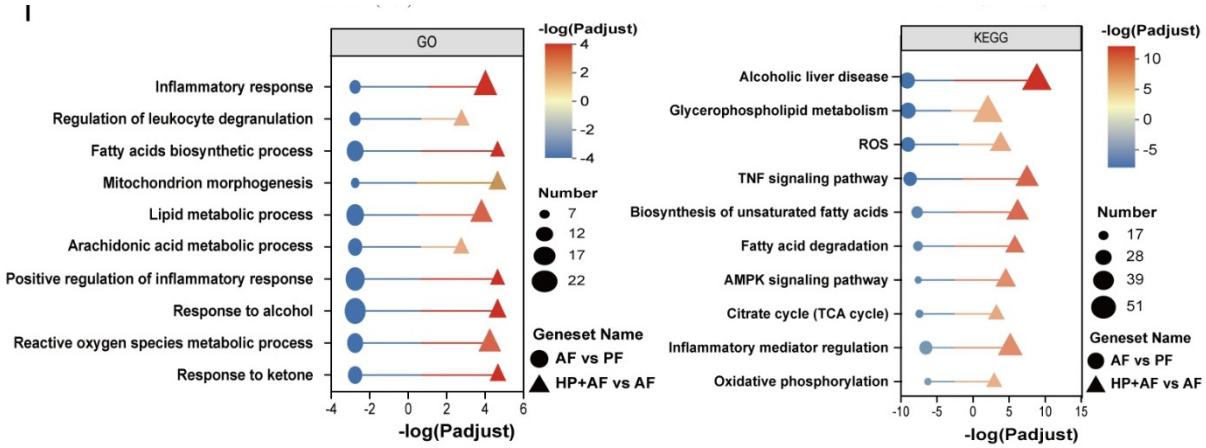


Figure 3. HP dietary pattern-protected ALD is associated with lipid metabolism, reactive oxygen species (ROS), inflammation, and ketone pathways. (A) Veen diagram showing the overlap of differentially expressed genes among PF, AF HP + AF, and HP + PF. (B) The number of differentially expressed genes between PF and AF groups, between AF and HP + AF groups, and between AF and LP + AF groups, respectively. Veen diagram showing the numbers of (C) HP diet reversed alcohol-unregulated genes, (D) HP diet reversed alcohol-downregulated genes, (E) LP diet aggravated alcohol-downregulated genes, (F) LP diet aggravated alcohol-upregulated genes, (G) HP diet rescued but LP diet aggravated alcohol-downregulated genes, (H) HP diet rescued but LP diet aggravated alcohol-upregulated genes. (I) Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses for differentially expressed genes between AF group vs. PF group and HP + AF *vs.* AF group. The data were originated from three independent liver samples for each group (n = 3).

3.3 HP dietary pattern reverses alcohol-induced disorder of lipid metabolism related genes

Based on transcriptome sequencing analysis, we observed that thirty-seven chronic alcohol-dysregulated lipid metabolism-associated genes were reversed by HP diet intervention (Fig. 4A). These genes are mainly clustered in lipogenesis, fatty acids β-oxidation, lipolysis, and fatty acids transmembrane inward transport signaling pathways. Therefore, classic targets in these pathways were selected for further validation. Our data showed that chronic alcohol-fed significantly: a) promoted lipogenisis *via* upregulating the mRNA levels of stearoyl-coA desaturase (SCD) and sterol regulatory element binding transcription factor 1 (SREBF1); b) stimulated fatty acid uptake *via* increasing very low density lipoprotein receptor (VLDLR) and cluster of differentiation 36 (CD36) expressions; c) blocked fatty acids catabolism *via* inhibiting peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (PPARGC1A), carnitine palmitoyltransferase 1 alpha (CPT1A), and peroxisome proliferator activated receptor alpha (PPARA) expressions; and d) promoted lipolysis *via* upregulating the expressions of diacylglycerol-O-acyltransferase homolog 2 (DGAT2) (Fig. 4B). Notably, HP diet intervention significantly improved alcohol-induced dysregulation of lipid metabolism associated targets mentioned above, along with some of them were aggravated in LP diet group (Fig. 4B).

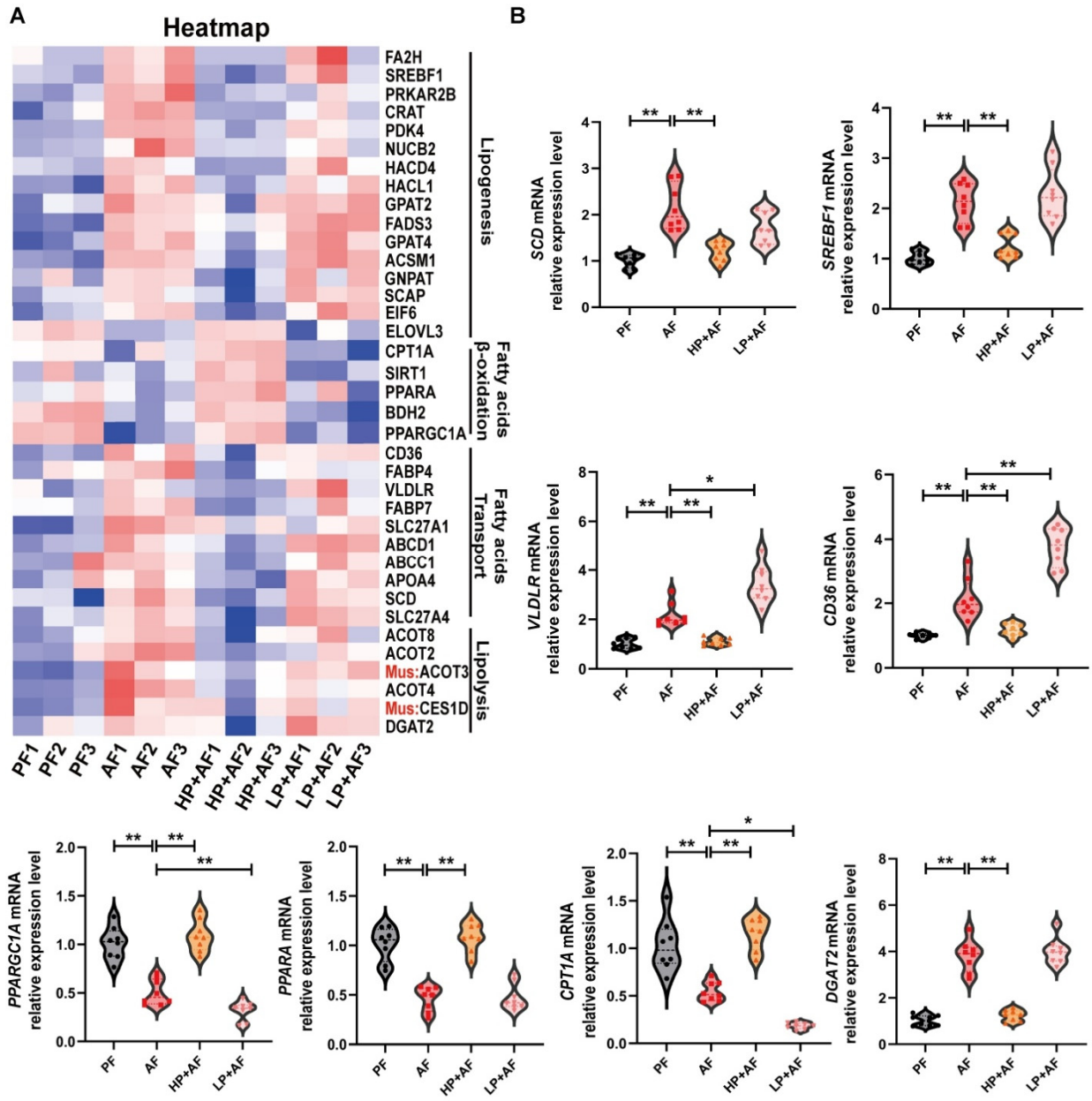


Figure 4. HP dietary pattern reverses alcohol-induced disorder of lipid metabolism related genes. (A) Clustering heatmap of differential gene enrichment analysis for liver lipid metabolism. (B) The mRNA levels of hepatic SCD, SREBF1, VLFLR, CD36, PPARGC1A, PPARA, CPT1A, and DGAT2. Data are presented as means ± SD (n = 8). *$P < 0.05$, and **$P < 0.01$ indicate statistically significant.

3.4 HP dietary pattern improves hepatic oxidative stress and down-regulates CYP2E1 in chronic alcohol-fed mice

In this study, several hepatic hallmark indicators of oxidative stress were assessed, since transcriptome sequencing analysis revealed that ROS pathway was closely associated with the protective role of HP diet. The results showed that chronic alcohol consumption significantly elevated the formation of MDA in the liver (Fig. 5A). Furthermore, the activities of antioxidant enzymes were disrupted by alcohol, evidenced by the reduced levels of SOD, GSH-Px, and GSH (Fig. 5B-D), along with increased GSSG levels and decreased GSH/GSSG ratio (Fig. 5E & F). Compared to AF group, all the tested indicators aforementioned were significantly ameliorated by HP diet, while LP diet further decreased GSH level and exacerbated the levels of

MDA and GSSG (Fig. 5). Ethanol catabolism, especially catalyzed by CYP2E1, has been well-documented contributing to the excessive production of ROS and further inducing oxidative stress. Therefore, the expression of CYP2E1 was detected in this study. Our data showed that chronic alcohol feeding strongly upregulated the mRNA levels of *CYP2E1*, which was robustly reversed by HP diet intervention, but was aggravated by LP diet treatment (Fig. S2A). Besides CYP2E1, transcriptome sequencing analysis revealed that seven genes related to alcohol metabolism were upregulated by chronic alcohol feeding, but were reversed by HP diet intervention (Fig. S2B).

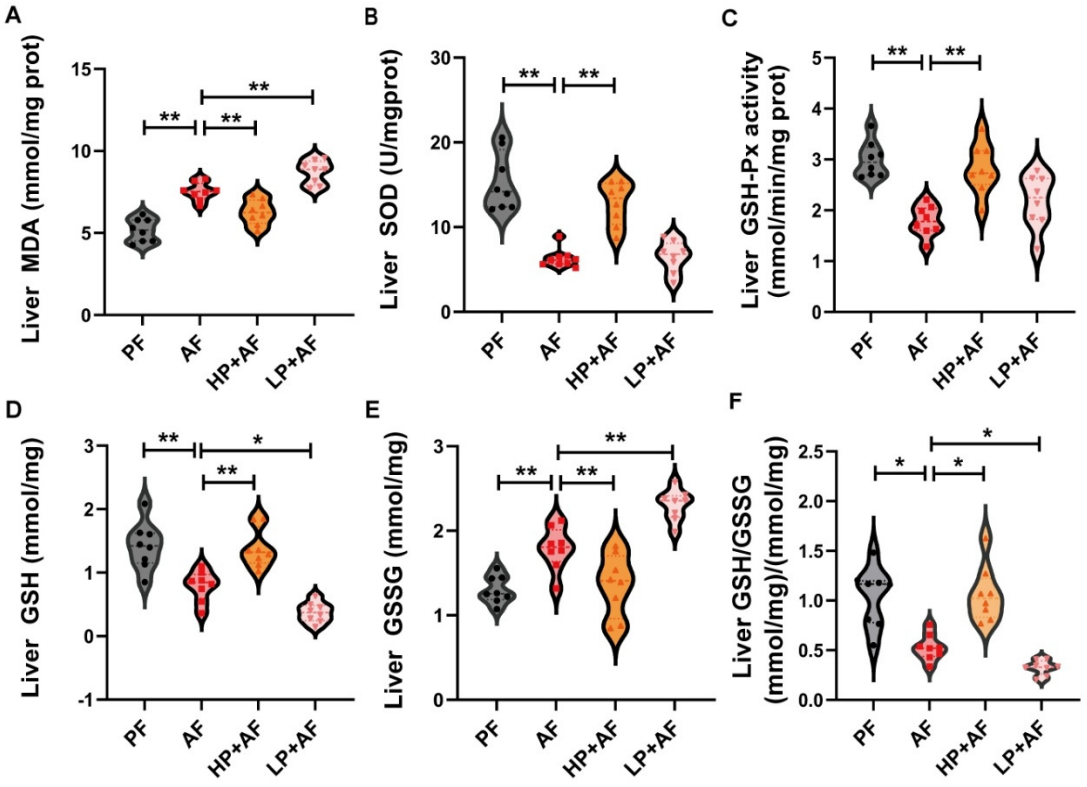


Figure 5. HP dietary pattern improves alcohol-induced hepatic oxidative stress in chronic alcohol-fed mice. (A) Liver MDA. (B) Liver SOD. (C) Liver GSH-px. (D) Liver GSH. (E) Liver GSSG. (F) GSH to GSSG ratio. Data are presented as means ± SD (n = 8). **P* < 0.05, and ***P* < 0.01 indicate statistically significant.

3.5 HP dietary pattern ameliorates alcohol-induced liver inflammation

It has been well-established that the elevation of proinflammatory factors contribute to the pathogenesis of alcohol-induced liver dysfunction. As shown in Fig. 6A, a total of 30 genes in the inflammatory signaling pathway were identified implicating in HP diet intervention-improved hepatic inflammation. Subsequently, three representative pro-inflammatory factors, including tumor necrosis factor (*TNF*), interleukin-1β (*IL1B*), and interleukin-6 (*IL6*) were verified by qRT-PCR. We observed that chronic alcohol consumption significantly increased the transcriptional levels of *TNF*, *IL1B*, and *IL6* when compared to that in the PF group (Fig. 6B). Notably, alcohol increased expressions of these pro-inflammatory cytokines was abolished by the HP diet intervention, but were aggravated in LP diet feeding mice liver (Fig. 6B).

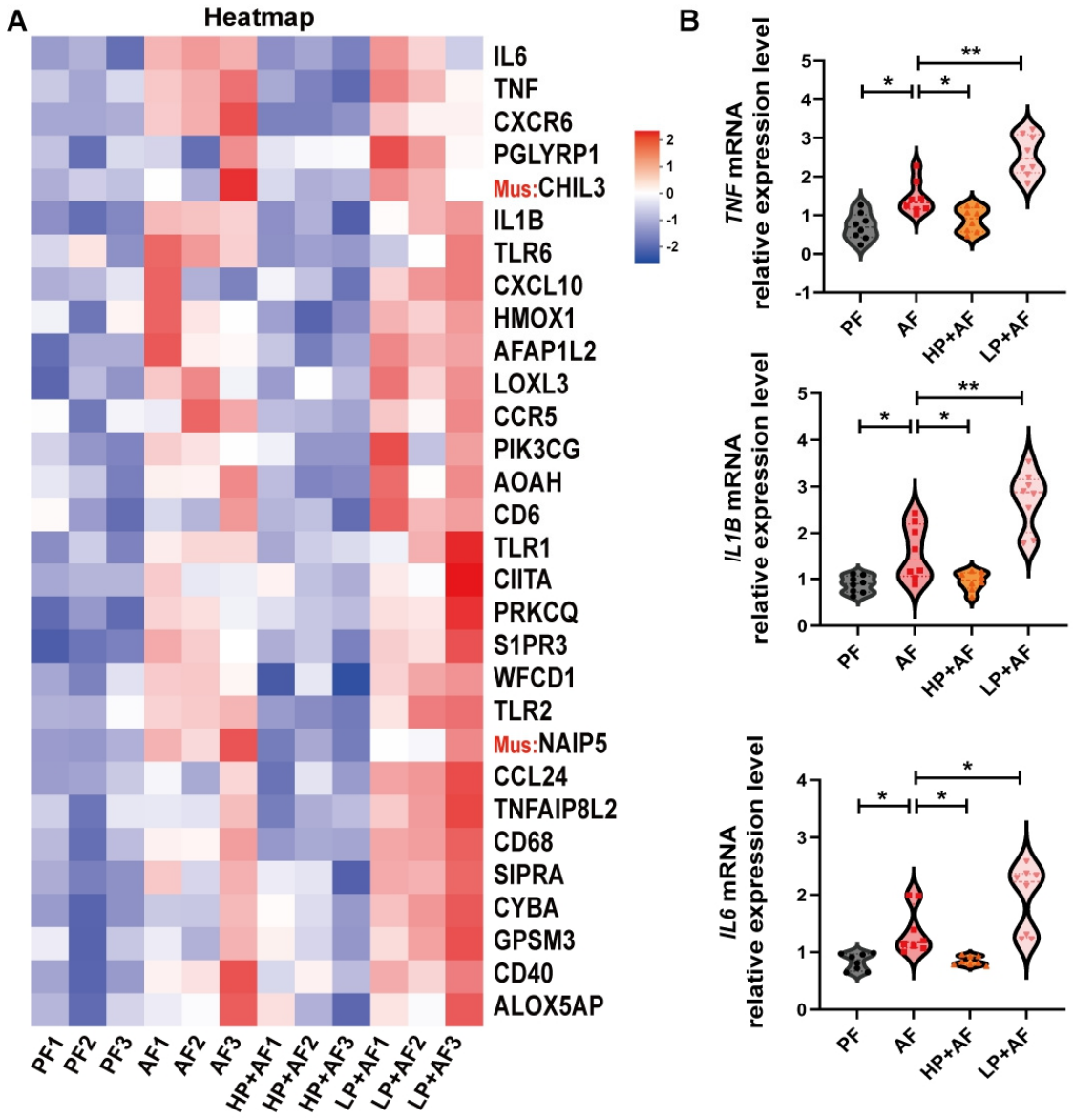


Figure 6. HP dietary pattern ameliorates alcohol-induced liver inflammation. (A) Clustering heatmap of differential gene enrichment analysis for inflammation. (B) The expressions of TNF, IL1B, and IL6 in mice liver were detected by qPCR. Data are presented as means ± SD (n = 8). **P* < 0.05, and ***P* < 0.01 indicate statistically significant.

3.6 HP dietary pattern reversed alcohol-induced dysregulations of Bdh2 and Morc4

As shown in Fig. 3G & H and Table S3, a total of eighty alcohol-disrupted genes, which were ameliorated by HP diet but were worsen by LP diet, were obtained by transcriptome sequencing analysis. Here, the top twenty differentially expressed genes were selected to explore the potential molecular targets involved in the protective role of HP diet. Among these genes, we observed that nine genes were up-regulated and eleven genes were down-regulated by alcohol consumption, respectively (Fig. 7A). The relationship between these genes and the biomarkers of hepatic steatosis and liver injury was examined using Spearman correlation coefficients. As shown in Fig.7B, biochemical indicators, including plasma ALT, AST, as well as hepatic and plasma TG and TC, were positively associated with most of the alcohol-stimulated genes, including *MORC4*, leptin receptor (*LEPR*), cytochrome P450 2A5 (*CYP2A5*), cytochrome P450 2A4 (*CYP2A4*), ATP binding cassette subfamily C member 4 (*ABCC4*), ATPase phospholipid transporting 8A1

(*ATP8A1*), CD84 antigen (CD84), cysteine sulfinic acid decarboxylase (*CSAD*) and myosin IXa (*MYO9A*), and negatively associated with *BDH2*, serine protease inhibitor E2 (*SERPINE2*), monooxygenase DBH like 1 (*MOXD1*), sulfotransferase family 1A member 1 (*SULT5A1*), carbonic anhydrase 1 (*CA1*), ribonuclease 2 (*RNASE2*), solute carrier organic anion transporter family member 1A1 (*SLCO1A1*), complement C4A (*C4A*), and carboxylesterases 3B (*CES3B*). The associations between the differentially expressed genes and the biomarkers related to oxidative stress, including MDA, SOD, GSSG, GSH, and GSH-Px were analyzed subsequently. Our data showed that the oxidative markers, including hepatic MDA and GSSG, were negatively correlated with the tested alcohol-downregulated genes, except for *A230005M16Rik* and cytochrome P450 7B1 (*CYP7B1*), and positively associated with all the alcohol-upregulated genes (Fig. 7B). While the antioxidant markers, including GSH, SOD, and GSH Px, were positively associated with most of the alcohol-downregulated genes, and negatively associated with alcohol-upregulated genes (Fig. 7B). Subsequently, qRT-PCR was utilized to verify the expression of aforementioned genes in the liver tissues. Our data confirmed that fourteen alcohol-disrupted genes were alleviated by HP diet intervention but were aggravated by LP diet treatment with *BDH2* and *MORC4* ranking on the top of alcohol-downregulated and -upregulated genes, respectively (Fig. 7C).

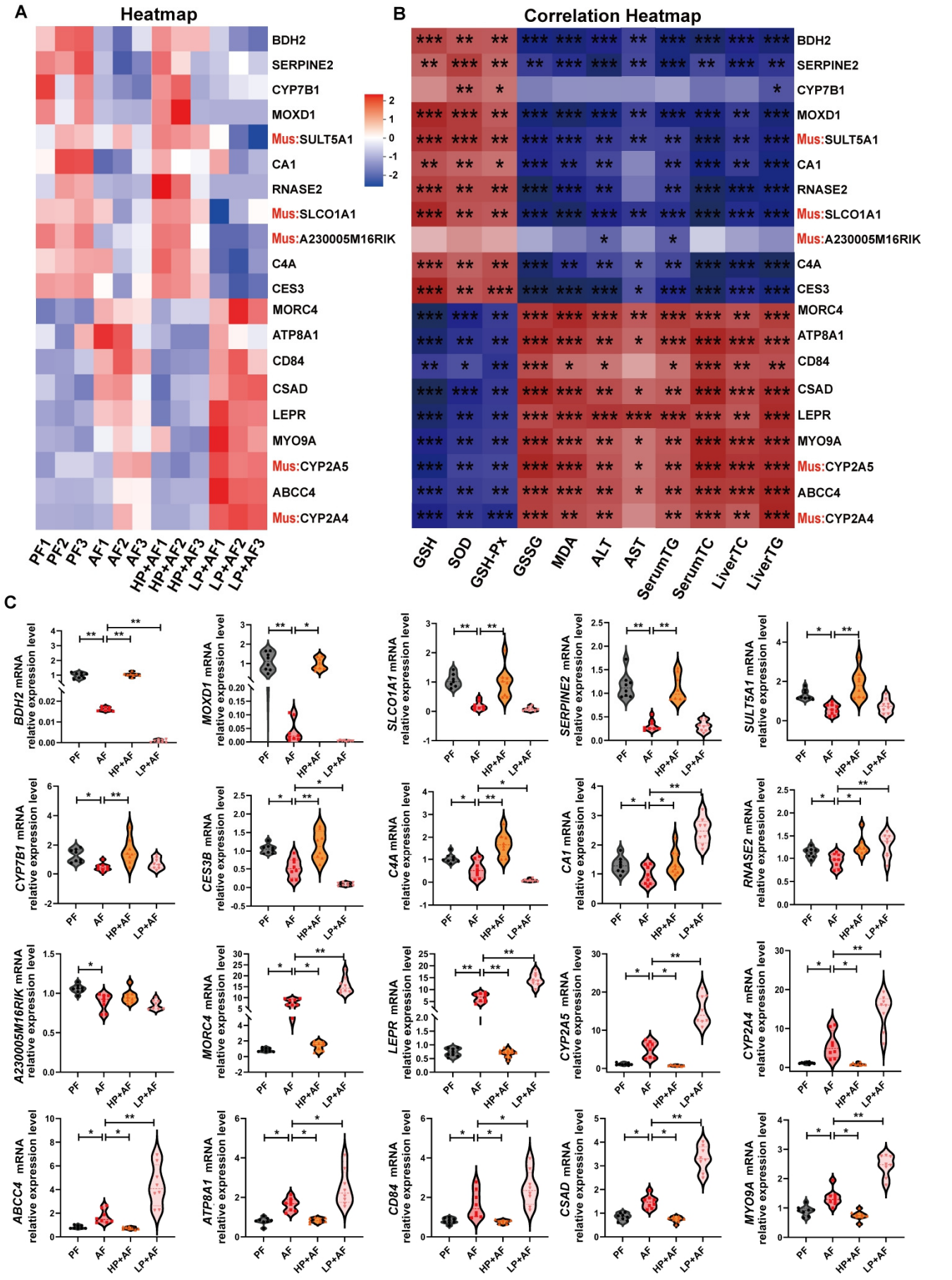
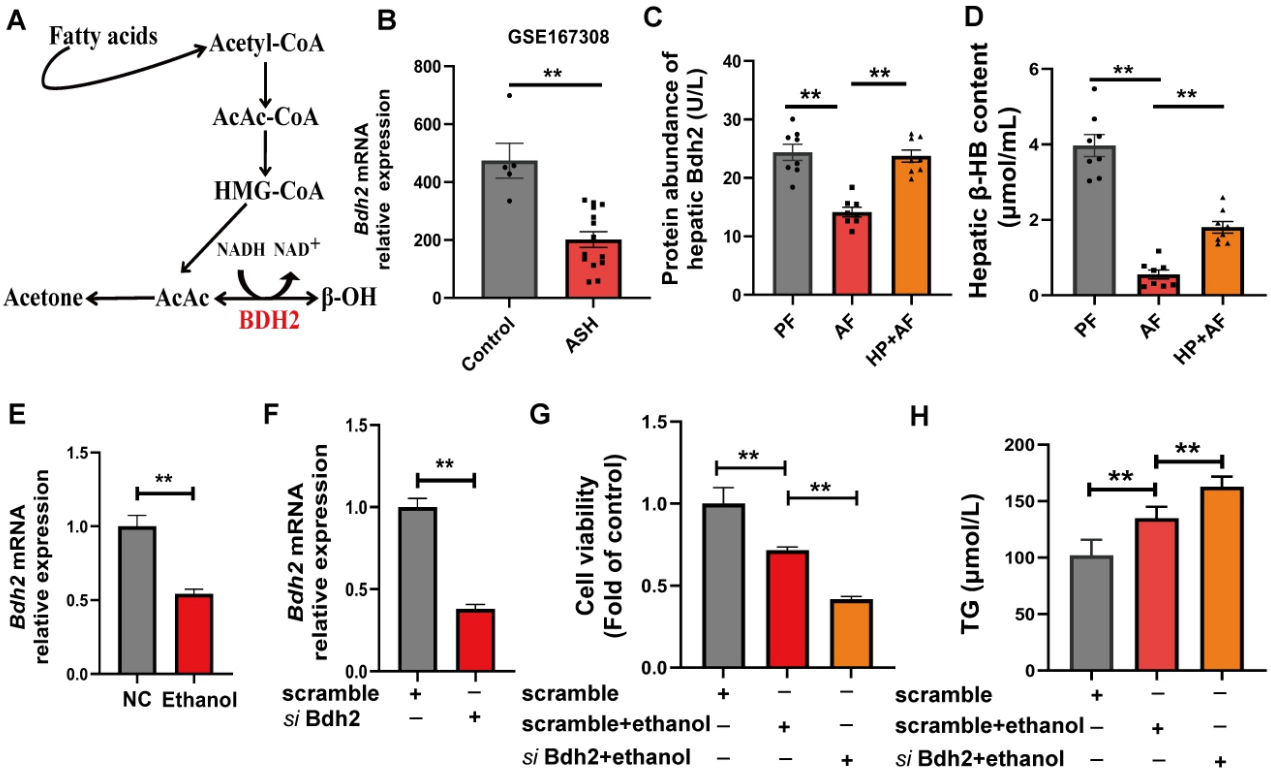


Figure 7. Twenty genes potentially involved in HP protection against alcohol induced liver injury. (A) Differential gene enrichment analysis clustering heatmap (n = 3). (B) Correlation analysis between differentially expressed genes and pathological parameters (n = 3). (C) mRNA expression of twenty regulated genes in mice liver tissue (n = 8). Data are presented as means ± SD. *$P < 0.05$, and **$P < 0.01$ indicate statistically significant.

3.7 Bdh2 is involved in alcohol-induced lipid accumulation and hepatotoxicity

The role of *Bdh2* and *Morc4* on alcohol-induced hepatocytes dysfunction was analyzed next. As shown in Fig. S3A, the mRNA level of *MORC4* was significantly upregulated in alcohol-exposed hepatocytes. While genetically silencing *MORC4* using its special *si*RNA did not block alcohol-induced cell death and intracellular lipid accumulation (Fig. S3B-E), preliminarily excluding the involvement of *MORC4* in the pathological process in ALD and its participation in HP diet-protected ALD. BDH2, a key enzyme in the ketogenic metabolic pathway, and mainly controlling the reversible reaction from acetoacetate (AcAc) to β-HB, was evaluated further (Fig. 8A). After analyzing the public data based on ALD human liver samples, we observed that the transcriptional levels of *BDH2* was significantly decreased in the patients (Fig. 8B). The protein abundance of BDH2 and the hepatic β-HB contents were detected by the commercialized kits, our data showed that HP diet intervention effectively reversed the reduction of BDH2 and β-HB in response to chronic alcohol consumption (Fig. 8C & D). In cultured hepatocytes, alcohol exposure markedly decreased the expression of *BDH2* (Fig. 8E). Genetically silencing *BDH2* by its special *si*RNA obviously aggravated alcohol-induced hepatocellular death and intracellular triglyceride deposition (Fig. 8F-I). Moreover, *BDH2* knockdown enhanced alcohol-induced excessive production of ROS, dysregulation of lipid metabolism-associated genes, CYP2E1 upregulation, and activation of pro-inflammatory factors (Fig 8J-M), implying that *BDH2* is a potential target participating in the pathological progress of ALD and might be mechanistically involved in beneficial role of HP diet.



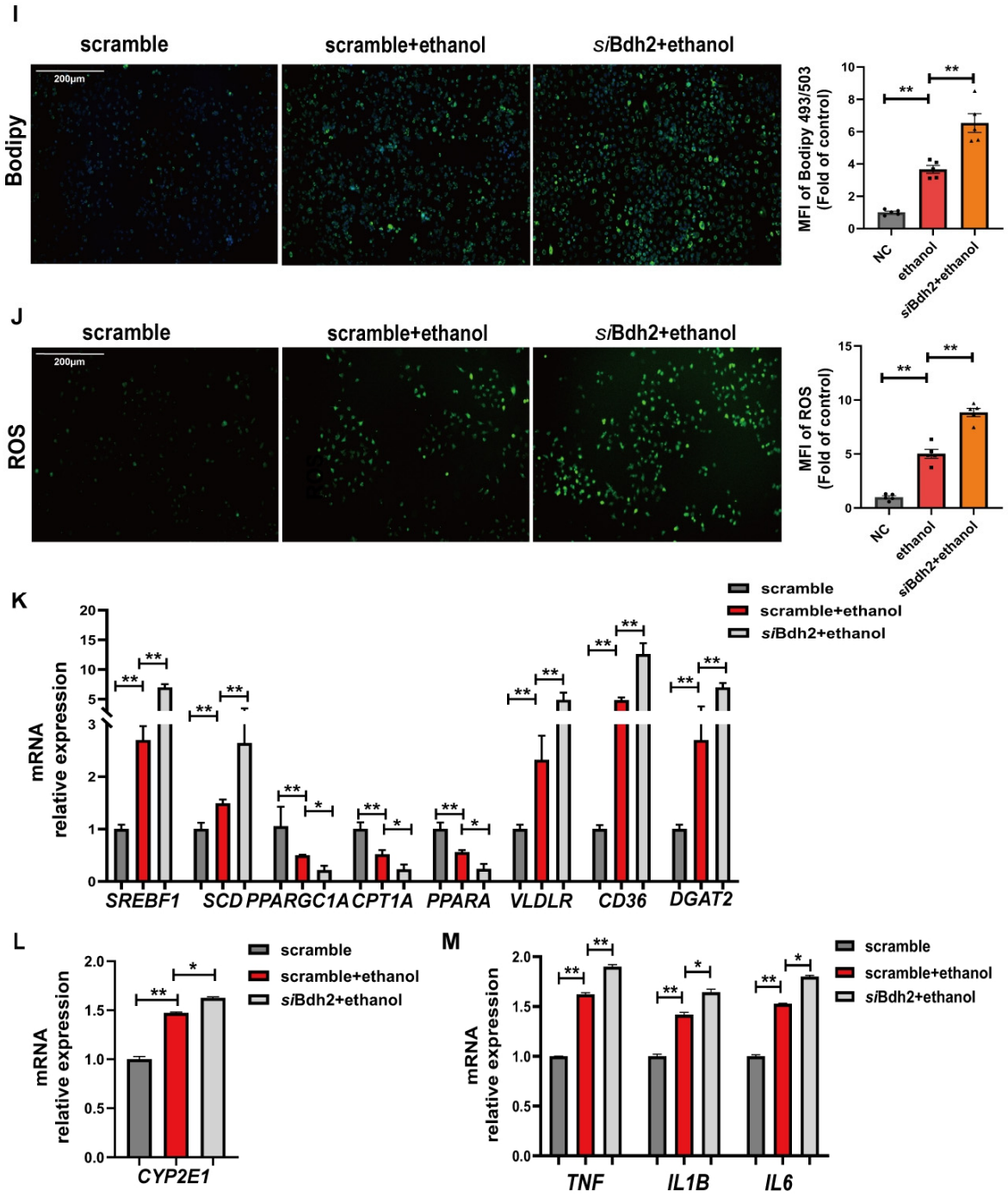


Figure 8. Bdh2 is involved in alcohol-induced lipid accumulation and hepatotoxicity. AML-12 mouse hepatocytes were treated with ethanol (100 mmol/L) for 16 h. Small inference RNAs were employed to silence Bdh2. Samples were collected for further detections. (A) Schematic diagram of BDH2 involvement in ketone metabolism. (B) Public data based on ALD human liver samples was employed to analyzed the transcriptional levels of Bdh2. (C) The protein abundance of hepatic BDH2. (D) Hepatic β-HB contents. (E) mRNA levels of Bdh2. (F) qRT-PCR was performed to analyze the knockdown efficiency of Bdh2. (G) Cell viability. (H) TG levels. (I) Bodipy 493/503 staining. (J) Detection of cellular ROS using DCFH-DA assay kit. (K-M) The effect of knocking down Bdh2 on RNA levels of SREBF1, SCD, PPARGC1A, CPT1A, PPARA, VLDLR, CD36, DGAT2, TNF, IL1B, IL6, and CYP2E1. Data are presented as means ± SD (n = 3-6). *$P < 0.05$ and **$P < 0.01$ indicate statistically significant.

4. Discussion

Our investigation provides initial evidence that isocaloric HP dietary pattern is an effective strategy to prevent against ALD. We demonstrated that HP diet intervention significantly reduced hepatic steatosis, liver injury, oxidative stress, and inflammation in response to chronic alcohol consumption in mice. Further

mechanistic study disclosed *BDH2*, a novel target that contributes to both the pathological progress of ALD and the protective role of HP diet.

Nutritional therapy has been proposed as a necessary measure to treat patients with ALD, since malnutrition is typically existed in this population. Emerging evidence showed the beneficial role of nutrients supplementation, such as vitamins (A, B1, B3, B6, B9, B12, C, D, and E), trace elements (zinc, iron, and selenium), animo acids (branched-chain amino acids, methionine, and glutamine), and foodborne functional factors on the improvement of ALD[13, 24, 25]. In recent years, the modification of macronutrients dietary pattern has been proven to be a promising strategy to defend against metabolic diseases, such as obesity, type 2 diabetes, and non-alcoholic fatty liver disease[14, 15, 26]. However, there is still a huge cognitive gap regarding macronutrients dietary pattern and ALD. Protein malnutrition is a common pathological feature of ALD, especially in the patients with advanced ALD[27]. The present study was conducted to evaluate the role of dietary protein proportion on ALD. In this study, the modification of macronutrients ratio is based on an isocaloric dietary pattern, that is, animal in each group consumes the same amount energy from the diet, to eliminate the influence of energy differences on the research results. In either PF or AF group, the protein accounted for 15% dietary energy, since this proportion meets the recommended standard of acceptable macronutrient distribution ranges (AMDR) of protein in the dietary reference intakes (DRIs) for most countries, and is a commonly used ratio in animal feed. The proportion of protein in HP diet group was set based on the observations from several clinical trials that 30% energy from protein has been reported to improve intrahepatic lipid deposition in the patients with various metabolic diseases[15, 18, 19, 21]. Additionally, a relatively mild LP (10% energy, the lower threshold of AMDR in DRIs for China and United States of America) group was employed to help systematically evaluating the effect of protein ratio on ALD. To the best knowledge of us, this study demonstrated for the first time that isocaloric HP diet pattern markedly ameliorated alcohol-induced hepatic steatosis and liver injury in mice, while LP diet aggravated ALD. Those results revealed the importance of dietary protein ratio on ALD and highlights the beneficial role of isocaloric HP diet protecting against ALD. Based on our team's prior research findings and relevant literature, the proportion of carbohydrate was adjusted to compensate the alteration of protein ratio in HP group with a fixed fat proportion in the diet. The reasons are as follows: (1) a previous human-based clinical trial has showed that isocaloric high protein low carbohydrate diet reduce liver fat and inflammation in individuals with type 2 diabetes using the same macronutrient energy distribution ratio as ours' HP diet[19]; (2) Accumulating evidence from both clinical investigations and experimental studies has consistently shown that a high-protein, low-carbohydrate diet exerts beneficial effects on fatty liver improvement[15, 18, 20, 28, 29]; (3) we previously reported that, under an isocaloric diet with a constant protein energy ratio, reducing the proportion of carbohydrates helps improve alcohol-associated liver disease[16]. Accordingly, we opted to increase the protein energy ratio by reducing the proportion of carbohydrates. However, whether lowing the proportion of fat in HP diet with a fixed carbohydrate ratio also improves ALD is still unclear. Future studies are needed to addressed this interesting issue.

Subsequently, transcriptomics was employed on the liver samples to elucidate the potential mechanisms behind HP diet-alleviated ALD. GO and KEGG analyses showed that the protective role HP diet was associated with lipid metabolism, ROS, inflammation, and ketone pathways. It is well-established that the body primarily metabolizes alcohol *via* ADH, a process that consumes NAD^+ to generate NADH. Chronic excessive alcohol consumption depletes hepatic NAD^+ levels, thereby impairing ADH-mediated alcohol metabolism. When ADH-mediated alcohol metabolism is compromised, feedback upregulation of ADH expression occurs – a phenomenon consistently observed in both chronic alcohol consumers and experimental animal models[30, 31]. Concurrently, as ADH capacity becomes saturated, the MEOS is upregulated, predominantly through increased CYP2E1 expression. Oxidative stress, induced by excessive intracellular ROS generated from ethanol catabolism by CYP2E1, has been well-addressed as a typically pathogenic cause of ALD[32, 33]. Oxidative stress leads to a dysregulation of multiple lipid-metabolism relative genes, and stimulates intrahepatic lipid accumulation, which further promotes the development of hepatic inflammation and liver injury[11]. Liver specific CYP2E1 knockout significantly improved alcohol-induced oxidative stress and further pathological alterations in ALD mice[34]. In our study, high-protein dietary intervention attenuated alcohol-induced upregulation of alcohol-metabolizing enzymes including ADH1, CYP2E1 and CYP1A1. We hypothesize this effect is mediated through restoration of hepatic NAD^+ levels. Supporting this, our data demonstrate that chronic alcohol exposure significantly reduced hepatic NAD^+ levels in experimental mice, while high-protein dietary intervention effectively reversed alcohol-induced NAD^+ decrease (data not shown). To further establish the causal relationship between hepatic NAD^+ levels and expression of alcohol-metabolizing enzymes (ADH1/CYP2E1), we administered nicotinamide (NAM), an NAD^+ precursor, to ALD mice[35]. Notably, NAM intervention similarly reversed alcohol-induced suppression of ADH1 and CYP2E1 expression (Fig. S4). These findings suggest that HP diet restores alcohol metabolic capacity by normalizing hepatic NAD^+ homeostasis, thereby modulating expression of key metabolic enzymes. Alcohol-induced oxidative stress, lipid metabolism-related genes dysregulation, and pro-inflammatory factors elevation were also dramatically alleviated by HP diet. Those data implied the involvement of an antioxidative mechanism of HP diet in the improvement of ALD *via* regulating CYP2E1.

To delineate the potential targets involved in HP diet-improved ALD, differentially expressed genes were screened by a rigorous statistical analysis and qPCR validation, and the top ranked genes were selected for further analysis. Our data revealed that HP diet intervention significantly reversed alcohol-downregulated *BDH2* and -upregulated *MORC4* in the liver, respectively. *MORC4* encoded protein MORC4, a nucleoplasm located protein containing a nuclear matrix binding domain, a N-terminal ATPase-like ATP-binding region and a CW four-cysteine zinc-finger motif, is widely distributed in various tissues, including the liver[36, 37]. It has been reported that MORC4 elevation is implicated in the pathogenesis of various cancers and inflammatory diseases[38-40]. Limited study has been conducted to disclose the role of MORC4 in ALD so far. Here, we observed that alcohol exposure significantly upregulated the transcriptional level of *MORC4* in both ALD mice liver and cultured hepatocytes. However, genetically knockdown *MORC4* could not effectively

reverse alcohol-induced hepatocytes death and lipid accumulation. Therefore, HP diet-reversed the upregulation of *MORC4* was not a primary molecular mechanism for HP diet-improved ALD.

The involvement of *BDH2* in HP diet-alleviated ALD was considered further. BDH controls the final step in hepatic ketogenesis process *via* catalyzing the reversible reaction of acetoacetate to β-hydroxybutyrate. Emerging evidence showed that BDH participates in various cellular biology processes, such as proliferation, cell cycle, apoptosis, fibrosis, and inflammation[41, 42]. The dysregulation of BDH is closely associated with various diseases, including cancer, diabetic cardiomyopathy, and metabolic dysfunction related liver disease[43-45]. Two subfamilies of BDH have been identified, including BDH1 and BDH2, while BDH2 is predicted locating in the cytosol and expressing in the liver and other organs[46]. To the best acknowledge of us, limited study has reported the role of BDH2 in ALD so far. In this study, we reported for the first time that: a) BDH2 was downregulated in the liver of ALD patients, ALD mice liver, and alcohol-exposed hepatocytes; b) alcohol-induced reduction of hepatic BDH2 in either abundance or activity was robustly rescued by HP diet intervention; c) *BDH2* expression is negative associated with liver steatosis, liver injury, and oxidative stress; d) genetically silencing *BDH2* significantly aggravated alcohol-induced hepatocytes death and intracellular lipid deposition, along with the overproduction of ROS, lipid metabolism-related genes dysregulation, and pro-inflammatory factors activation. These results collectively implied the involvement of BDH2 in HP diet-improved ALD. Although the exact mechanisms behind BDH2-regulated liver dysregulation in ALD are not fully understood, β-HB, the metabolite of BDH2, might be contributing to BDH2-improved ALD. Since in this study, we observed a markedly decrease of β-HB in ALD mice liver. In support of us, previous studies have reported the abatement of β-HB in the liver of both patients and experimental animals with ALD[47, 48]. Importantly, it has been reported that β-HB supplementation protected chronic alcohol-induced hepatic steatosis, liver injury, and hepatic oxidative stress and inflammation[47]. In this study, we found that HP diet intervention significantly reversed alcohol-induced decline in hepatic β-HB, implying the involvement of BDH2-regulated β-HB in the beneficial role of HP diet.

There are several limitations in this study. a) casein is the only source of protein in the feed, how about protein from other sources affects ALD is deserved to be investigated; b) the role of *BDH2* in response to alcohol exposure was only evaluated in cultured hepatocytes, the application of animal model with liver specific *BDH2* knockout will consolidate the evidence; c) Besides *BDH2*, the involvement of other transcriptomics-discovered differential genes in HP diet-improved ALD should be considered; d) the dysfunction of muti-organs, including the enhancement of lipolysis in adipose tissue and the impairment of intestinal barrier function, has been reported to be implicated in the pathological progress of ALD, whether HP diet improves these pathological changes is also worth studying in the future; e) the precise mechanism by which HP diet regulates hepatic NAD^+ metabolism remains an intriguing open question; f) only a single sex (male) was employed in this study. Although this study did not specifically examine the effects of a HP diet intervention on ALD in female mice, clinical trials have reported that HP diet are effective against fatty liver in both male and female of tested patients[49, 50]. Additionally, pharmaceutical intervention (e.g., lycium

barbarum polysaccharide) that demonstrate ameliorative effects in males also exert comparable therapeutic benefits in females[51], implying that HP diet might likewise have preventive effects on ALD in females. Given that both human and animal studies have demonstrated that females are more susceptible to ALD[52, 53], evaluating whether HP diet mitigates fatty liver in female drinkers or experimental ALD model remains an important and clinically relevant question for future research.

Conclusion

We demonstrate that isocaloric HP dietary pattern improves chronic alcohol-induced intrahepatic lipid accumulation and liver injury in C57BL/6N mice. Further mechanistic investigation indicates that *Bdh2* is a potential therapeutic target of ALD, contributing to the protective role of the HP diet. Our findings indicate that isocaloric HP dietary pattern may be a promising strategy for improving ALD.

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author, upon reasonable request.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (U24A20767, 82273625, 82103838) and Zhejiang Natural Science Foundation (RG25H260001).

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