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Role of anethole dithiolethione regulating liver lipid metabolism, oxidative and ER stress in NAFLD: Insights from a mouse model

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Abstract: In preliminary studies, Anethole Dithiolethione (ADT) has exhibited significant potential in reducing the accumulation of reactive oxygen species (ROS) and regulating mitochondrial fusion (Mfn) in relation to non-alcoholic fatty liver disease (NAFLD). This study sought to investigate the specific role of ADT in NAFLD using a mouse model, with C57BL/6J mice divided into four groups: normal diet, high-fat diet (HFD), HFD with 10 mg/kg ADT, and HFD with 30 mg/kg ADT. Pathological changes were assessed through oil red O and hematoxylin-eosin staining. Lipidomics profiling was conducted to determine the composition of phospholipids and real-time reverse transcription polymerase chain reaction and western blotting were utilized to analyze gene and protein expression related to liver phospholipid transport, endoplasmic reticulum (ER) stress and lipid synthesis. The results revealed that ADT increased the levels of phospholipid components such as phosphatidylserine and phosphatidylethanolamines, as well as the expression of relevant genes associated with liver lipid metabolism and ER stress (Mfn2, ATF6, PTDSS1, PTDSS2 and PPARα). ADT also demonstrated the ability to decrease levels of liver inflammatory indicators and oxidative stress induced by the HFD, including ALT, AST, IL-6, TNF-α, MDA and catalase. These findings suggest that ADT shows promise as a potential treatment for NAFLD by regulating Mfn2 expression and promoting phosphatidylserine transfer.

Keywords: Anethole Dithiolethione, Mitochondrial fusion protein 2, Non-alcoholic fatty liver disease, Phosphatidylserine

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a prevalent chronic liver disease，which ranges from benign steatosis to nonalcoholic steatohepatitis (NASH) andeven leading to cirrhosis and liver cancer (Zhang and Yang, 2021). In addition, NAFLD is associated with a higher risk for cardiovascular diseases, cancer and all-cause mortality (Lee *et al.*, 2022), affecting up to one third of the world's adult population. Despite the development of potential drugs for NAFLD targeting various processes, ideal drugs have not been approved for NAFLD or NASH at present (Harvey, 2022; Shi and Fan, 2022).

One promising approach for treating NAFLD is the use of hydrogen sulfide (H2S), an endogenous signaling gasotransmitter that exerts anti-apoptotic, anti-oxidative, and anti-inflammatory effects (Wang *et al.*, 2020). ADT, a sustained-release H2S donor, is used as a hepato-protective and choleretic drug in clinical practice. In preliminary studies, ADT has shown significant reduction in the accumulation of reactive oxygen species (ROS) and regulation of mitochondrial fusion state (MFN) (Zhao *et al.*, 2020), as well as the normalization of liver pathological changes induced by a high-fat diet (HFD).

One key player in the pathogenesis of NASH is mitochondrial fusion protein 2 (Mfn2), which plays a major role in mitochondrial fusion and the maintenance of bioenergetic function (Casellas-Díaz *et al.*, 2021). Recent research has revealed that Mfn2 deficiency is linked to reduced transfer of phosphatidylserine (PS) from the ER to mitochondria, resulting in reduced PS synthesis, ER stress, inflammation, and a NASH-like phenotype (Hernández-Alvarez *et al.*, 2019). On the other hand, over-expression of Mfn2 has been shown to attenuate the detrimental effects of excess exogenous free fatty acids (FFA) by improving mitochondrial function and decreasing the release of reactive oxygen species (ROS)(Dong *et al.*, 2020).

While it has been established that ADT plays a role in regulating the accumulation of reactive oxygen species (ROS) and maintaining mitochondrial fusion, its connection with Mfn2 (Mitofusin 2) in the development of Non-Alcoholic Steatohepatitis (NASH) is not yet clear (Zhao *et al.*, 2020). Our hypothesis suggests that ADT may have a role in the prevention and treatment of NAFLD by influencing the transportation and synthesis of PS (phosphatidylserine) through the regulation of Mfn2. If our hypothesis is confirmed, it could signify significant advancements in various fields. This includes confirming Mfn2 as a target protein for NAFLD, overcoming the

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limitations of AAV (Adeno-Associated Virus) vector regulation of Mfn2 in clinical applications and potentially discovering a new class of pro-drugs for H2S (Hydrogen Sulfide) to prevent and treat NAFLD. Confirmation of our hypothesis would bring about substantial progress in our understanding of the mechanisms underlying NAFLD and open up new avenues for therapeutic interventions.

MATERIALS AND METHODS

Animals and treatments

C57BL/6 mice were obtained from Hunan SJA Laboratory Animal Co., Ltd (SCXK Xiang 2019-0004). The mice were housed in the IVC cages at the Animal Experimental Center of the Third Xiangya Hospital, Central South University, under controlled environmental conditions. The temperature ranged from 22°C to 25°C, with a relative humidity of 50%±10% and a 12/12 hour day/night cycle. The mice had ad libitum access to water and food. All animal experiments were conducted in accordance with the guidelines of the Laboratory Animal Care and Welfare Committee of Central South University (Approval No. 2020sydw8013).

After a week of acclimatization, 24 mice were randomly divided into four groups: normal diet (0.5% CMC-Na and 0.4% soybean lecithin, purchased from Shanghai yuanye Bio-Technology Co., Ltd and Shandong Liaocheng Ahua Pharmaceutical Co., Ltd.), high-fat diet (HFD, customized by Ruidi Biotechnology Co., Ltd.), HFD+10 mg/kg ADT (purchased from Dalian Meilun Biotech Co., Ltd) and (HFD+30 mg/kg ADT, respectively.

ADT and/or vehicle were administered to the mice via gavage once a day for a duration of 10 weeks, as determined by previous studies. Before euthanasia, the mice were fasted overnight and then euthanized by decapitation after administration of chloral hydrate anesthesia the next morning. Blood samples were collected from the eyeballs and body weight, white fat, and liver weight were measured after sacrificing. The blood samples were left at room temperature for 1 hour before centrifugation at 3500 rpm for 10 minutes. The serum was then separated and stored at -80°C until further analysis. The left lobe of the liver was fixed in a 4% paraformaldehyde solution, while the remaining tissue was stored at -80°C after quick-freezing with liquid nitrogen.

Analysis of liver histology

Liver tissue samples were fixed in a 4% paraformaldehyde solution and then prepared as paraffin sections. Haematoxylin and eosin (H&E) and oil red O (ORO) staining were performed on the sections to examine hepatic steatosis and inflammation under light microscopy. Two pathologists blindly evaluated the extent of histopathological changes.

Biochemical analysis

Serum glutamate alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using an automatic biochemical analyzer (Hitachi 7600-210). The levels of TC, TG, MDA, SOD, GSH, IL-6 and TNF-α in liver homogenate were determined using standard laboratory detection kits according to the manufacturer's instructions.

Determination of phospholipid

The LC-MS-based lipidomics platform involves multiple steps, including sample preparation, separation, detection in positive ion mode, and data analysis.

Sample Preparation and Separation: 30mg of liver homogenate sample was mixed with 200μL H2O, 80μL methanol and 400μL MTBE. The mixture was vortexed for 60 seconds and ultrasonicated for 10 minutes in ice. The sample was centrifuged for 15 minutes at 3000 rpm. The upper phase (200μL) was collected and vacuum dried at 35°C. 150μL of chloroform and methanol (1:1, v/v) was added to the dried sample. Detection and Analysis: The temperature of the column was set to 35°C. 2μL of lipid extract was injected. Lipid compounds were detected in positive-ion mode using an Agilent 6490 QQQ mass spectrometer based on MRM mode. ESI ion source parameters were set as follows: dry gas temperature (200°C) of ion source (Gas Temp); nitrogen flow (12 L/min); sheath gas flow rate (12 L/min); sheath gas temperature (350°C); capillary voltage (4000 V). Data was processed using the Mass Hunter workstation quantitative analysis software and the method of the external standard.

Real-time quantitative PCR

Total RNA was extracted from frozen liver tissue using Trizol reagent and was reverse-transcribed into cDNA using the EVO M-MLV Reverse Transcription Kit II according to the manufacturer's instructions. RT-qPCR was performed using TB Green Premix Ex Taq ™ II (TliRNaseH Plus) and the Light Cycler 96 system. Nine genes involved in liver phospholipid transport, endoplasmic reticulum stress and lipid synthesis were detected, including Mitofusin2, PTDSS1, PTDSS2, SREBP1c, FAS, ACC, ATF4, ATF6 and CHOP. The gene-specific primers for mice are listed in table 1. The expression of target genes was normalized to that of GAPDH and relative quantification of mRNA levels was performed using the 2-ΔΔCt method.

Western blot

Total protein was extracted from liver fragments using homogenizing buffer containing protease inhibitors. Equivalent quantities of total protein resuspended in SDS-containing sample buffer were heated for 5 minutes at 100°C, separated by SDS-PAGE and transferred to pre-activated PVDF membranes. The PVDF membrane was sealed with 5% skim milk for 1 hour, followed by

incubation with specific primary antibodies at 4°C overnight and corresponding secondary antibodies at room temperature for 1 hour. Immunoreactive bands were visualized using ECL and quantified using software Image J.

The following antibodies were used: Anti-Mfn2 (1:2000, 12186-1-ap, Proteintech), anti-PTDSS1 (1:2000, ARP47068-P050), and anti-PTDSS2 (1:2000, ARP49960-P050) were provided by Aviva Systems Biology; anti-SREBP1c (1:2000, ab28481, Abcam), anti-elF2α (1:2000, ab169528, Abcam), and anti-PPARα (1:750, ab61182, Abcam) were purchased from Abcam; anti-FAS (1:500, 10624-2-ap, Proteintech), anti-ACC (1:3000, 21923-i-ap, Proteintech), anti-β-actin (1:5000, 66009-1-Ig, Proteintech) and anti-ATF6 (1:1000, 24169-1-ap, Proteintech) were purchased from Proteintech; anti-p-elF2α (1:1000, A#3398, CST), anti-CHOP (1:1000, 2895S, CST) were provided by Cell Signaling Technology; anti-ATF4 (1:200, sc-390063, purchased from Santa Cruz).

STATISTICAL ANALYSIS

The data were presented as mean±SD. Statistical analysis was conducted using one-way ANOVA, with P-values below 0.05 considered statistically significant. The data were analyzed using SPSS 26.0 software (IBM).

RESULTS

Histological study

After 10 weeks of intervention, differences were observed in the relative weight between the control, HFD, and ADT-treated groups (fig. 1). The HFD group showed a significant increase in the relative weight of the liver and white fat. However, the ADT-treated groups had significantly lower HFD-induced relative liver and white fat weight. H&E staining revealed significant lipid deposition and moderate blood stasis formation in the blood vessels, as well as local focal infiltration of lymphocytes in the HFD group. In contrast, lipid vacuoles were significantly decreased in the ADT-treated groups, with the hepatic structure returning to a morphologically normal state (fig. 2). Furthermore, ORO staining showed that ADT treatment visibly reduced the number of intracellular lipid droplets and attenuated HFD-induced hepatic lipid accumulation. Compared with the control group, TC and TG increased by 3-5 times and 2-3 times, respectively, in the HFD group, and the lipid accumulation was improved after mice were treated with ADT (P<0.05). There was no significant difference between high and low dose groups.

Liver inflammatory damage

Serum ALT and AST are important indicators of liver injury. The study also measured serum ALT, AST, IL-6, and TNF-α levels to evaluate the protective effect of ADT on HFD-induced liver damage. The results in fig. 3 showed that ALT and AST levels were significantly increased in the HFD group, and after ADT intervention, the levels of ALT and AST caused by HFD were significantly reduced (P<0.05). Moreover, the levels of IL-6 and TNF-α in the HFD group were significantly higher than the control group (P<0.05). However, when the mice were treated with ADT, the levels of these inflammatory factors significantly decreased in the ADT-treated group (shown in fig. 3). The results indicated that ADT played a protective role against HFD-induced hepatic damage by decreasing the levels of inflammatory mediators.

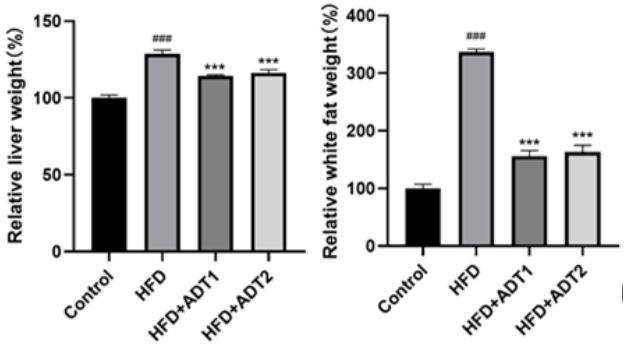


Fig. 1: Relative liver weight and Relative white fat weight at the end of the experiment (g, n=6, 10week). Data are expressed as mean±SD, *### P<0.001 versus Control group; *** P<0.001 versus HFD group.

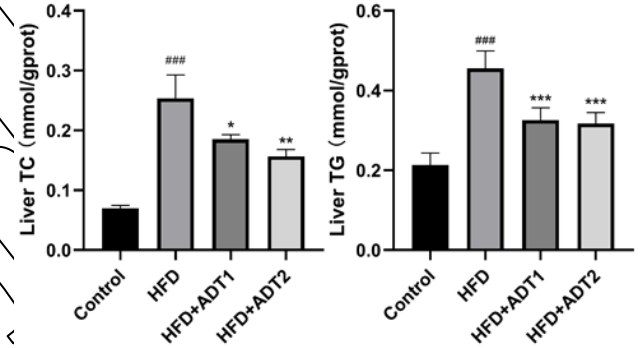


Fig. 2: The effect of ADT on liver lipid accumulation in mice (n = 6)

Oxidative stress

In addition, fig. 4 demonstrated that compared with the control group, the level of malondialdehyde (MDA) in the HFD group was significantly increased, while the superoxide dismutase (SOD) and reduced glutathione (GSH) levels were significantly decreased. However, administration of ADT could increase the activity of SOD to maintain the high level of GSH and eliminate MDA, effectively reversing the oxidative stress injury induced by HFD.

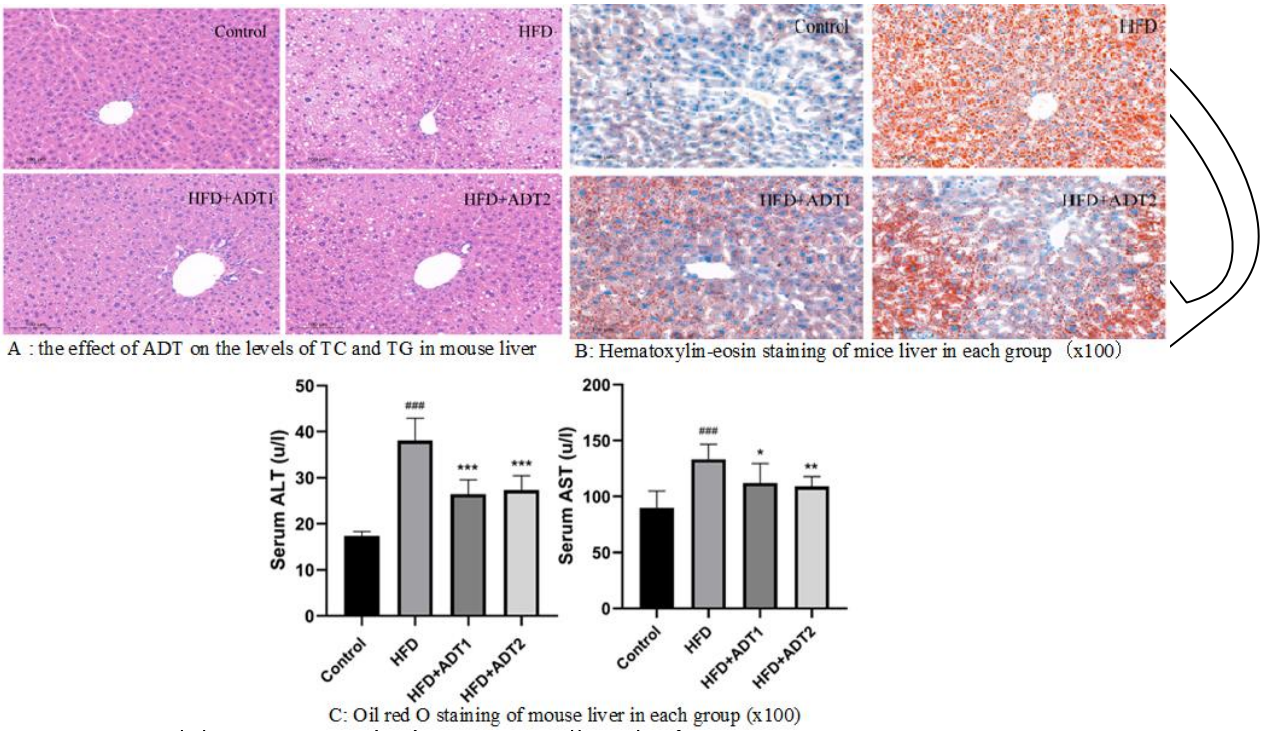
Phospholipid metabolism and ER-mitochondrial phosphatidylserine transfer

In mammalian cells, phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are metabolically related membrane

Table 1: Primer sequences used for real-time quantitative PCR

Gene	Forward primer	Reverse primer
Mfn2	5'-CTTCGTGTCTGCCAAGGAGGTTC-3'	5'-GCCGCTCTTCCCGCATTTCTAG-3′
PTDSS1	5'-CTACACGAGAAGCGGACATCATGG-3′	5'-CCAGCCAGATTCCACCACCATTG-3′
PTDSS2	5'-GCAGCCATCACAGTCACAGAGC-3′	5'-CCTCAGCAGTCACACCGTCATTG-3′
SREBP1c	5'-TGGAGGCAGAGAGCAGAGATGG-3′	5'-TGGAGCAGGTGGCGGATGAG-3′
FAS	5'-CGGCTGCGTGGCTATGATTATGG-3′	5'-GTGAGGTTGCTGTCGTCTGTAGTC-3′
ACC	5'-CAACATTCGCCTGACAACAACTGG-3′	5′-GGACTGTGCCTGGAACCTCTTTG-3′
ATF4	5'-CGGCTATGGATGATGGCTTGGC-3′	5'-GGAATGCTCTGGAGTGGAAGACAG-3′
ATF6	5'-CATCTCCTCTCCTCGGTCCACAG-3′	5'-AAAGGCTTGGGCTGAACTGAAGG-3′
CHOP	5'-CTACTCTTGACCCTGCGTCCCTAG-3′	5'-TCGTTCTCCTGCTCCTTCTCCTTC-3′
GAPDH	5'-TCACCATCTTCCAGGAGCGAGAC-3′	5'-TGAGCCCTTCCACAATGCCAAAG-3'

Abbreviations: Mitochondrial fusion protein 2 (Mfn2), Phosphatidylserine synthase 1 (PTDSS1), Phosphatidylserine synthase 2 (PTDSS2), Sterol-regulatory element-binding protein 1c (SREBP1c), Fatty acid synthase (FAS), Acetyl-CoA carboxylase (ACC), Activating transcription factor 4 (ATF4), Activating transcription factor 6 (ATF6)



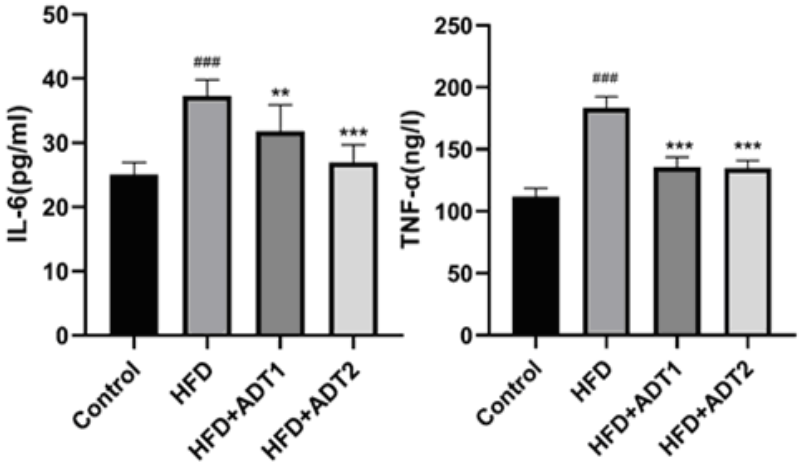
Data are expressed as mean±SD , *### P<0.001 versus Control group ; *** P<0.001 versus HFD group.

Fig. 3: The anti-inflammatory effect of ADT in mice (n = 6)

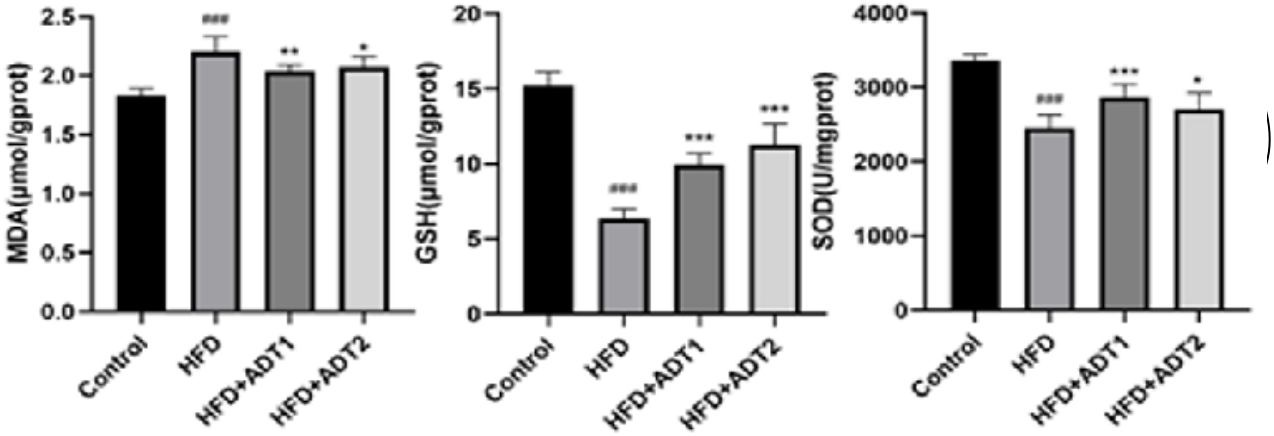
aminophospholipids. PS is synthesized in the ER membranes from PC through the enzyme PTDSS1 and from PE through the enzyme PTDSS2. fig. 5 showed that the levels of phosphatidylserine (PS) and phosphatidylethanolamine (PE) were significantly decreased (P<0.001) in the HFD group, while the phosphatidylcholine (PC) level was slightly decreased (P<0.05). However, when the mice were treated with ADT, PS, PE and PC levels were significantly increased. In addition, HFD significantly decreased the expression of Mfn2, PTDSS1 and PTDSS2 in the liver (shown in fig. 6-A), which are related to phospholipid synthesis and transformation. However, ADT could reverse this trend compared to the HFD group, as demonstrated in fig. 6.

ER stress and lipid metabolism

Excessive ER stress triggered the PERK/eIF2α/ATF4/CHOP axis, which promoted lipid metabolism disorders and contributed significantly to the pathogenesis of NAFLD. The mRNAs and proteins related to the signaling pathway and β-oxidation were measured, as seen in fig. 6-A. Compared with the control group, mice in the HFD group displayed ER stress with high mRNA and protein expression of PERK/eIF2α, ATF4, CHOP, SREBP-1c, FAS, and ACC, as well as low ATF6 and PPARα expression (P<0.001). However, ADT treatment could reduce the over-expression of PERK/eIF2α, ATF4, CHOP, SREBP-1c, FAS and ACC, while increasing the ATF6 and PPARα expression (P<0.001).



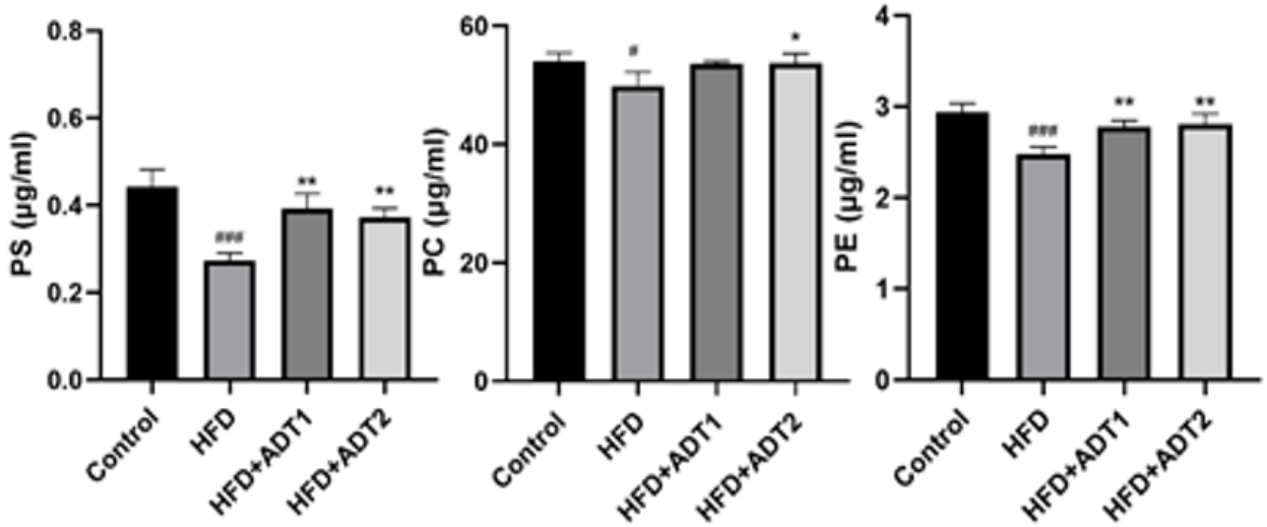
A : The effect of ADT on the levels of ALT and AST in mouse liver



B: The effect of ADT on the levels of IL-6、TNF-α in mouse liver (n = 6)

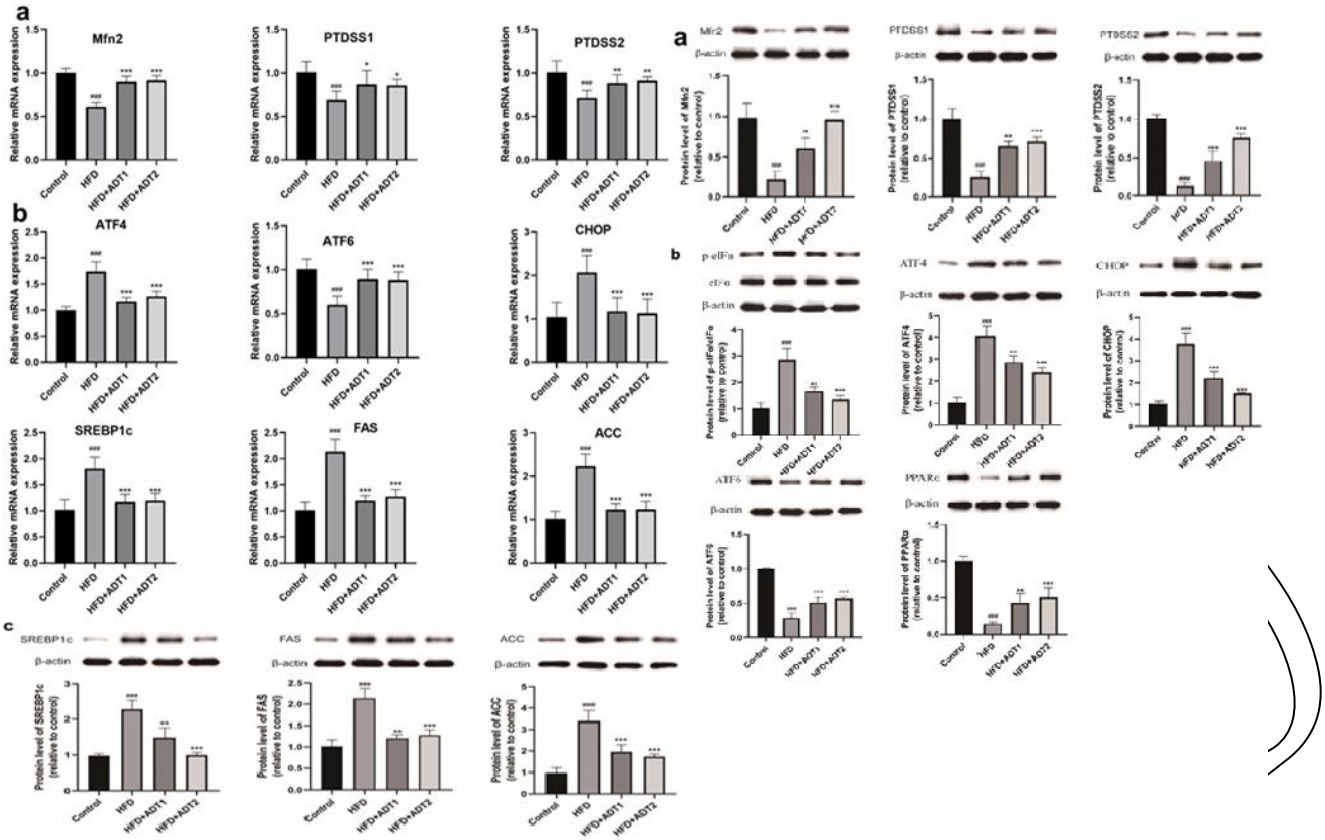
Data are expressed as mean±SD , *### P<0.001 versus Control group ; *** P<0.001 versus HFD group.

Fig. 4: The effect of ADT on the levels of SOD, GSH and MDA in mouse liver (n=6)



Data are expressed as mean±SD , *### P<0.001 versus Control group; *** P<0.001 versus HFD group.

Fig. 5: The effect of ADT on the phospholipid components in mouse liver (n = 3) (n=6)



A: Related mRNA; B:Related proteins (a) Phospholipid transport synthesis: Mfn2, PTDSS1, PTDSS2; (b) endoplasmic reticulum stress: ATF4, ATF6, CHOP; (c) Lipid and fatty acid synthesis: SREBP1c, FAS, ACC. Data are expressed as mean±SD, *### P<0.001 versus Control group; *** P<0.001 versus HFD group.

Fig. 6: The levels of related genes mRNA and proteins in mouse liver (n = 6)

DISCUSSION

In our study, we have confirmed that Mfn2 is a critical protein involved in the initiation and progression of NAFLD. Importantly, we have reported, for the first time, that intervention with ADT has beneficial effects on various aspects of NAFLD, including liver lipid accumulation, structural abnormalities, liver inflammation, oxidative stress, phospholipid metabolism, and ER stress. We propose that the mechanism underlying ADT's potential for NAFLD treatment involves the regulation of Mfn2 and ER-mitochondrial phosphatidylserine transfer.

Mfn2 is a crucial mitochondrial membrane protein in mitochondrial autophagy, mitochondrial motility, lipid transfer across mitochondria-ER contacts (Zaman and Shutt, 2022). When Mfn2 expression is reduced, not enough PS is imported into mitochondria for decarboxylation to PE. Obstacles in PS decarboxylase bring about mitochondrial defects, which causes abnormal β-oxidation and oxidative phosphorylation, leading to oxidative stress, inflammation, cell death and apoptotic pathways (Li *et al.*, 2022). In the study, Mfn2, PTDSS1, and PTDSS2 levels were significantly down regulated in the HFD mouse models, which could be reversed by ADT. The results showed that ADT upregulated the expression of Mfn2, PTDSS1 and PTDSS2 and maintained mitochondrial metabolism, insulin signaling, and lipid homeostasis. When homeostasis is disrupted, abnormal phospholipid components of the endoplasmic reticulum membrane are effective activators of the unfolded protein response (UPR). The UPR regulates gene expression in response to endoplasmic reticulum stress (ERS), which activates eukaryotic translation initiation factor 2 subunit alpha (eIF2α) kinase and protein kinase R-like ER kinase (PERK). EIF2α phosphorylation can protect hepatocytes from oxidative stress by maintaining endogenous antioxidant levels and controlling ROS-defense gene expression to restore cellular homeostasis. EIF2α dephosphorylation leads to hepatic steatosis and hepatocyte apoptosis by alleviating ER stress (Tang *et al.*, 2020). Endoplasmic reticulum stress triggers cell injury and apoptosis through PERK-ATF4-CHOP pathways (Li *et al.*, 2020). Our study indicated that HFD treatment increased the expression of ERS-related proteins, including p-ERK/eIF2α, ATF4, CHOP, while ADT significantly decreased these elevated

proteins. ATF6 is a transcription factor of the unfolded protein response, which is an upstream regulator of fatty acid metabolism and proteostasis (Glembotski *et al.*, 2020). ATF6 over-expression enhances the transcriptional activity of peroxisome proliferator-activated receptor-α (PPARα). PPARα, a master regulator of lipid metabolism in the liver, can promote fatty acid β-oxidation (FAO) and oxidative phosphorylation (OXPHOS)(Yang *et al.*, 2021). The transcription factor SREBP1c, which triggers the expression of downstream genes involved in lipid biosynthesis, such as Acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), can be suppressed by PPARα enhancing Insig2a gene expression (Nguyen *et al.*, 2021). It is widely accepted that a high-fat diet (HFD) triggers the activation of ATF6 and PPARα, which in turn promotes fatty acid β-oxidation to maintain a balanced lipid metabolism during the initial stages of endoplasmic reticulum stress. However, when an excessive amount of fat surpasses the cellular capacity for β-oxidation, this delicate balance is disrupted and leads to the persistent presence of endoplasmic reticulum stress. Consequently, lipotoxic damage occurs in the liver.

Our study has elucidated the interaction between proteins and lipids, which are key components of the cell membrane. These interactions play a vital role in modulating the function of hepatic cells dependent on the membrane, influencing various properties such as antioxidant activity, anti-inflammatory response, anti-fibrotic effects and cellular signaling pathways (Hernández-Alvarez *et al.*, 2019). By understanding these protein-lipid interactions, we can gain insights into the underlying mechanisms of NAFLD and explore potential therapeutic targets and strategies.

Our study has overcome several limitations in previous research. Firstly, instead of using intravenous administration of adenoviruses encoding Mfn2, we have screened for a small molecule drug that can regulate Mfn2 expression. This approach offers a more practical and feasible option for potential therapeutic interventions. Secondly, we have highlighted the potential of ADT as a promising drug candidate due to its ability to target multiple aspects of NAFLD, including preventing steatosis and reducing inflammation. Lastly, our study suggests a novel perspective on the use of pro drugs of H_2S, which may represent a new class of drugs for the prevention and treatment of NAFLD.

In this study, it is worth noting that we did not observe fibrosis in the liver tissues of the model mice. This limitation is consistent with previous reports in the literature. However, it is important to acknowledge that fibrosis is a critical aspect of NAFLD progression, and our study did not specifically address the anti-fibrotic effects of H2S. In future experiments, it would be beneficial to extend the duration of the high-fat diet in the model mice to induce fibrosis and assess the potential anti-fibrotic effects of H2S.Based on existing literature, it is reasonable to speculate that H2S may have an anti-fibrotic effect due to its ability to inhibit inflammatory factors. Chronic inflammation plays a significant role in the development of fibrosis and H2S has been reported to possess anti-inflammatory properties (Zhang *et al.*, 2015). However, it is important to note that further experimental verification is necessary to confirm the potential anti-fibrotic effect of H2S.

CONCLUSION

In conclusion, our study provides valuable insights into the role of Mfn2 in the initiation and progression of NAFLD and highlights the potential therapeutic effects of ADT in treating NAFLD. We have demonstrated that ADT intervention can effectively mitigate various aspects of NAFLD, including liver lipid accumulation, structural abnormalities, inflammation, oxidative stress, phospholipid metabolism and ER stress. These beneficial effects are likely mediated through the regulation of Mfn2 and ER-mitochondrial phosphatidylserine transfer.

Furthermore, our study has shed light on the intricate protein-lipid interactions that are crucial for modulating the function of hepatic cells. By understanding these interactions, we have identified potential targets and strategies for the development of novel therapeutic interventions for NAFLD.

Overall, our findings contribute to the growing body of knowledge on the pathogenesis of NAFLD and offer new possibilities for therapeutic interventions. Further research is warranted to fully understand the mechanisms underlying Mfn2 and ADT's effects on NAFLD and to explore the potential of H2S as a therapeutic agent for NAFLD treatment.

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