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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 regulating mitochondrial fusion protein (Mfn) and mitigating the buildup of reactive oxygen species (ROS) in relation to NAFLD. This study aimed to explore the distinct role of ADT in the context of NAFLD by employing a mouse model. The C57BL/6J mice were divided into four groups: a regular diet group, a high-fat diet (HFD) group, and two groups receiving HFD supplemented with either 10 or 30 mg/kg ADT. Pathological changes were assessed through oil red O and hematoxylin-eosin staining. Lipidomics profiling was conducted to ascertain the composition of phospholipids, and RT-PCR along with WB were employed to analyze gene and protein expression pertinent to liver phospholipid transport, endoplasmic reticulum (ER) stress and lipid synthesis. The findings indicated that ADT elevated the levels of phospholipid components such as phosphatidylserine (PS) and phosphatidylethanolamine (PE), along with the upregulation of genes associated with liver lipid metabolism and endoplasmic reticulum (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 imply that ADT may serve as a promising therapeutic intervention for NAFLD by regulating Mfn2 expression and promoting PS transfer.

Keywords: Anethole dithiolethione, mitochondrial fusion protein 2, non-alcoholic fatty liver disease, phosphatidylserine

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INTRODUCTION

NAFLD has emerged as a prevalent chronic liver condition, particularly in the context of effectively managed hepatitis C virus (HCV) and hepatitis B virus (HBV) infections. It is crucial to prevent NAFLD from progressing to cirrhosis and hepatocellular carcinoma (Zhang and Yang, 2021). In addition, NAFLD is linked to an increased risk of cardiovascular diseases, cancer and all-cause mortality, as reported by Lee *et al.* (Lee *et al.*, 2022). 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 (H_2S), an endogenous signaling gasotransmitter that possesses properties such as inhibiting apoptosis, reducing oxidative stress, and curbing inflammation. (Wang *et al.*, 2020). ADT, a sustained-release H_2S 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 ROS and regulation of mitochondrial fusion state (MFN) (Zhao *et al.*, 2020), as well as the normalization of liver pathological changes induced by a HFD.

One key player in the pathogenesis of NASH is mitochondrial fusion protein 2 (Mfn2), which is instrumental in the process of mitochondrial fusion and in sustaining the energy metabolism (Casellas-Díaz *et al.*, 2021). Recent studies have revealed that Mfn2 deficiency is linked to decreased transfer of PS from the ER to mitochondria, resulting in diminished 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 adverse impacts of excess exogenous free fatty acids by enhancing mitochondrial performance and reducing the emission of ROS (Dong *et al.*, 2020).

Although the involvement of ADT in modulating ROS accumulation and sustaining mitochondrial fusion is acknowledged, its connection with Mfn2 (Mitofusin 2) in the development of NASH remains to be elucidated (Zhao *et al.*, 2020). Our hypothesis suggests that ADT could potentially contribute to the prevention and management of NAFLD by influencing the transport and synthesis of PS through the regulation of Mfn2.If our hypothesis is confirmed, it could signify significant advancements in

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various fields. This includes confirming Mfn2 as a target protein for NAFLD, overcoming the limitations of AAV (Adeno-Associated Virus) vector regulation of Mfn2 in clinical applications and potentially discovering a new class of pro-drugs for H_2S (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

Animal grouping and intervention

C57BL/6 mice, sourced from Hunan SJA Laboratory Animal Co., Ltd (SCXK Xiang 2019-0004), were accommodated in individually ventilated cages at the Animal Experimental Center, affiliated with the Third Xiangya Hospital. These mice were under controlled environmental conditions. The temperature ranged from 22°C to 25°C and the relative humidity carefully balanced at 50%±10%, adhering to a 12/12-hour light/dark cycle. The mice enjoyed unrestricted access to both food and water throughout the study. All experimental procedures involving the animals were performed in strict accordance with the guidelines set by the Laboratory Animal Care and Welfare Committee of Central South University, having obtained prior approval (Approval No. 2020sydw8013).

Twenty-four mice were randomly assigned to four separate experimental groups after a one-week adaptation period. The control group was administered a standard diet comprising 0.5% sodium carboxymethyl cellulose (0.5%) and soybean lecithin (0.4%), both ingredients sourced 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.

The mice were administered ADT and/or vehicle via gavage once daily for a duration of 10 weeks, as determined by previous studies. Before euthanasia, the mice were subjected to an overnight fast and humanely euthanized through decapitation after administration of chloral hydrate anesthesia the next morning. Blood samples were promptly collected via ocular puncture and subsequent measurements of body weight, white adipose tissue, and liver mass were conducted post-sacrifice. The blood samples were allowed to coagulate at ambient temperature for one hour prior to being centrifuged at revolutions per minute for a duration of 10 minutes to obtain serum. The serum was aliquoted and stored at -80° C for future analysis. Meanwhile, the left hepatic lobe was fixed in a paraformaldehyde solution (4%) for histological examination, while the remaining liver tissue was quickly frozen in liquid nitrogen and kept at -80C for subsequent use.

Liver histopathological examination

Liver specimens were harvested and immersed in a 4% paraformaldehyde solution for fixation. The tissues were stained using hematoxylin and eosin as well as oil red O stains, which were performed to assess hepatic steatosis and inflammation under light microscopy. Two pathologists blindly evaluated the extent of histopathological changes.

Biochemical analysis

Serum levels of AST and ALT were quantified utilizing an automated biochemical analysis system (Hitachi 7600-210). The levels of TC, TG, MDA, SOD, GSH, IL-6 and TNF-α in hepatic homogenate were determined utilizing standardized diagnostic kits, adhering to the protocols outlined by the manufacturers.

Quantification 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 H_2O, 80μL methanol and 400μL MTBE. The mixture was vortexed for 60 seconds and ultrasonicated for 10 minutes while kept on ice to maintain low temperatures. The sample was centrifuged at 3000 rpm for 15 minutes to separate the components. The upper phase (200μL) was collected and vacuum dried at 35°C. 150μL of methanol and chloroform, in a 1:1 volume ratio, was added to the dried sample.

Detection and Analysis: The temperature of column was adjusted 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. The parameters for the electrospray ionization (ESI) source were meticulously configured as follows: dry gas temperature (200°C) of ion source (Gas Temp); sheath gas temperature (350°C); capillary voltage (4000V); sheath gas flow rate (12 L/min); nitrogen flow (12 L/min). The data underwent quantitative analysis using quantitative analysis software (the Mass Hunter work station) and the method of the external standard.

Real-time quantitative PCR

Total RNA was extracted from frozen liver tissue using Trizol reagent following the manufacturer's protocol. This RNA was subsequently reverse-transcribed into complementary DNA (cDNA) through the application of the EVO M-MLV Reverse Transcription Kit II. RT-qPCR was executed utilizing TB Green Premix Ex Taq II 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 levels of these target genes

were standardized against the expression of the GAPDH gene and to ensure an accurate assessment of gene expression changes, the relative quantification of mRNA levels was determined employing the 2- Δ Δ Ct method.

Western blot

Total proteins were extracted from liver fragments using a homogenizing buffer supplemented with protease inhibitors. Equivalent quantities of these proteins were then resuspended in an SDS-containing sample buffer and heated for 5 minutes at 100ºC. Subsequently, the proteins were resolved via SDS-PAGE electrophoresis and then transferred onto pre-activated PVDF membranes. These membranes were blocked with a 5% skim milk solution for one hour to prevent non-specific binding. After blocking, the membranes were incubated with specific primary antibodies overnight at 4ºC to allow for antibody-antigen interaction. The next day, the membranes were incubated with corresponding secondary antibodies for one hour at room temperature to amplify the signal. The immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) detection system, and their intensities were quantified using the ImageJ software for accurate analysis.

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-ACC (1:3000, 21923-i-ap, Proteintech), anti-β-actin(1:5000, 66009-1-Ig, Proteintech), anti-ATF6 (1:1000, 24169-1-ap, Proteintech) and anti-FAS (1:500, 10624-2-ap, Proteintech) were purchased from Proteintech; anti-p-elF2α (1:1000, A#3398, CST) and anti-CHOP (1:1000, 2895S, CST) were provided by Cell Signaling Technology; anti-ATF4 (1:200, sc-390063, purchased from Santa Cruz).

STATISTICAL ANALYSIS

Quantitative data in the study are presented as mean ± standard deviation (SD). For statistical comparisons, one-way analysis of variance (ANOVA) was employed, with statistical significance defined as P-values less than 0.05. The data analysis was facilitated by the use of SPSS software, version 26.0 (IBM).

RESULTS

Histological study

Following a 10-week intervention period, significant variations were noted in the relative weight and hepatic accumulation among the control, high-fat diet (HFD), and ADT-treated groups, as depicted in Figs. 1 and 2. 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 stark contrast, the ADT-treated groups exhibited a marked reduction in lipid vacuoles, with the liver morphology reverting to a normal appearance (Fig. 3a). Additionally, ORO staining revealed that ADT treatment significantly decreased the quantity of intracellular lipid droplets and mitigated the hepatic lipid accumulation induced by a high-fat diet (fig. 3b). In comparison to the control group, total cholesterol (TC) and triglyceride (TG) levels were elevated by 3-5 times and 2-3 times, respectively, in the high-fat diet (HFD) group. Notably, lipid accumulation was ameliorated following ADT treatment in mice ($P<0.05$). No significant differences were noted between the groups that received high and low doses of ADT.

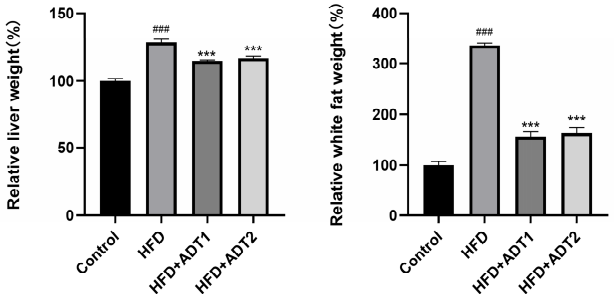


Fig. 1: Relative liver weight and Relative white fat weight at the end of the experiment (n=6, 10week), Data are expressed as mean±SD, ### $P<0.001$ versus Control group; *** $P<0.001$ versus HFD group.

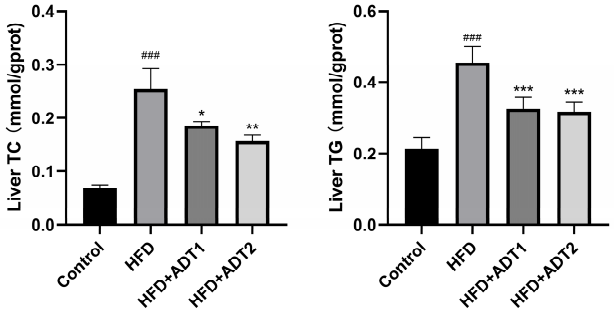


Fig. 2: The effect of ADT on liver lipid accumulation in mice (n = 6), Data are expressed as mean±SD, ### $P<0.001$ versus Control group; ***$P<0.001$ versus HFD group.

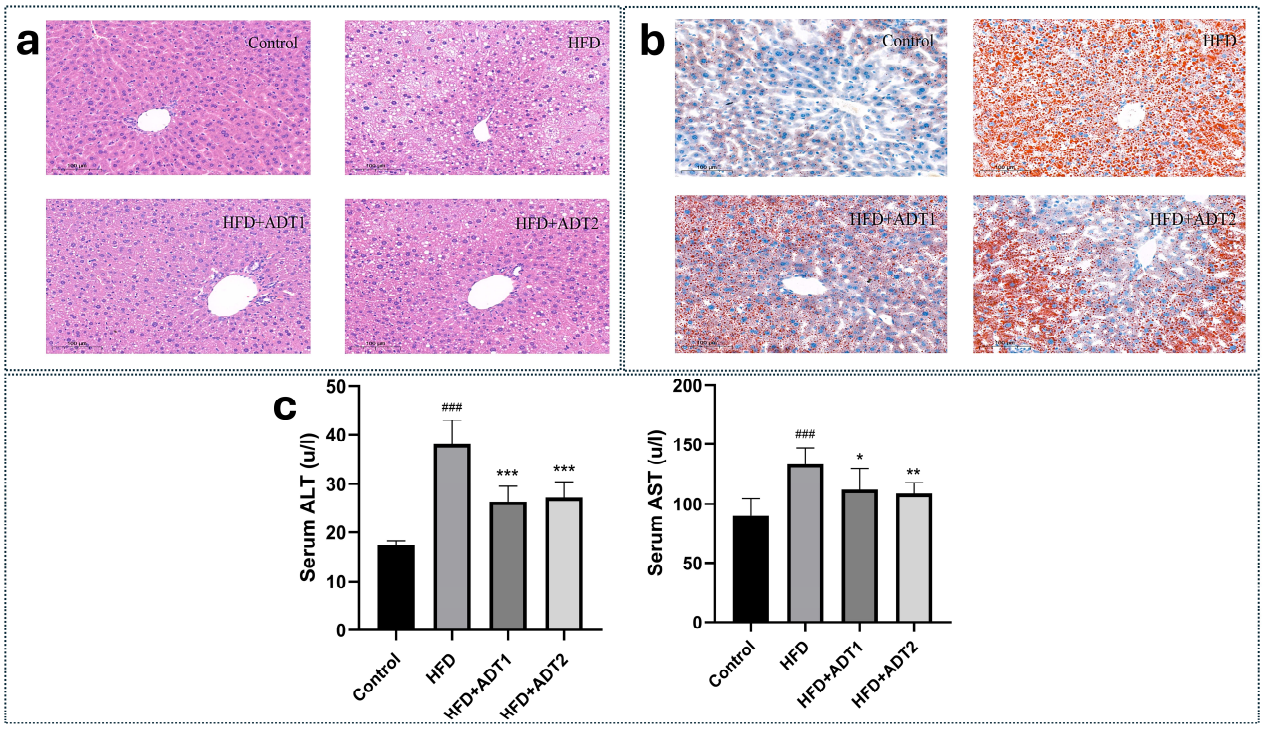
Liver inflammatory damage

ALT and AST in serum are pivotal indicators for assessing liver damage and for evaluating liver injury. This study assessed the protective effect of ADT on high-fat diet (HFD)-induced liver damage by measuring serum concentrations of ALT, AST, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). The findings presented in Fig. 3c revealed a statistically significant increase in serum ALT and AST levels in the HFD group. Following ADT intervention, these levels were significantly reduced, achieving statistical significance

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), (a) hematoxylin-eosin staining of mice liver in each group (x 100), (b) oil red O staining of mice liver in each group (x 100) (c) Effect of ADT on ALT and AST

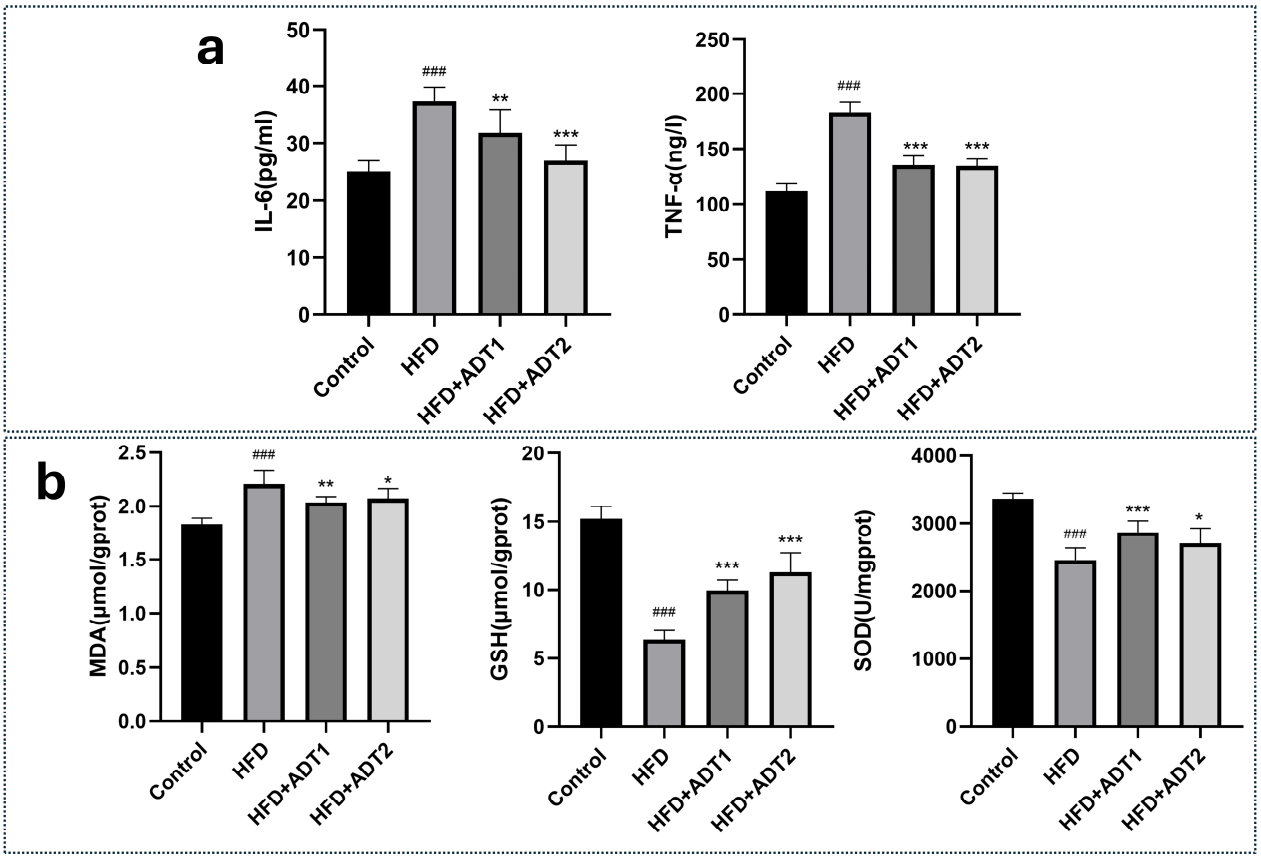
(P<0.05). Furthermore, the HFD group showed significantly higher levels of IL-6 and TNF- α compared to the control group, with statistical significance (P<0.05). Upon ADT treatment, the levels of these inflammatory cytokines were significantly lowered in the ADT-treated group, as depicted in fig. 4a. These results suggest that ADT exerts a protective effect against HFD-induced hepatic injury by reducing the levels of inflammatory mediators.

Oxidative stress

Furthermore, Fig. 4b depicts a significant surge in malondialdehyde (MDA) concentrations and a marked decrease in the levels of superoxide dismutase (SOD) and reduced glutathione (GSH) in the high-fat diet (HFD) group when compared to the control group. However, the administration of ADT was found to enhance SOD activity, preserve high levels of GSH, and eliminate MDA, effectively counteracting the oxidative stress damage caused by the HFD.

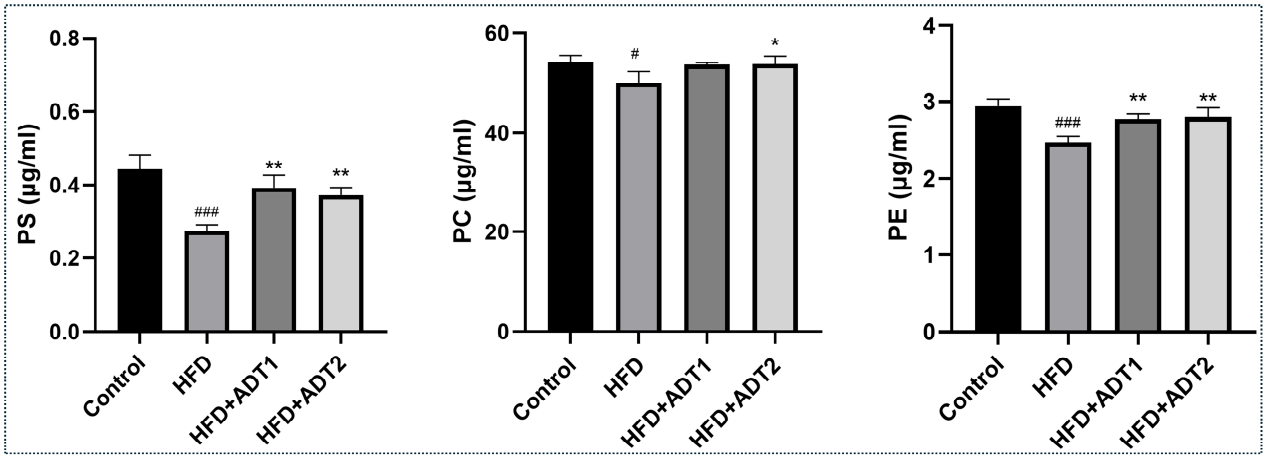
Phospholipid metabolism and ER-mitochondrial PS transfer

In mammalian cells, aminophospholipids, including PE, PS and Phosphatidylcholine (PC), are crucial for a variety of cellular processes. PS is synthesized in the ER membranes from PC through the enzyme PTDSS1 and from PE through the enzyme PTDSS2. Fig. 5 delineates a statistically significant decrement in the concentrations of PS and PE within the HFD group (P<0.001).



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 (a) IL-6 and TNF-α (b) SOD, GSH and MDA in mouse liver (n=6).



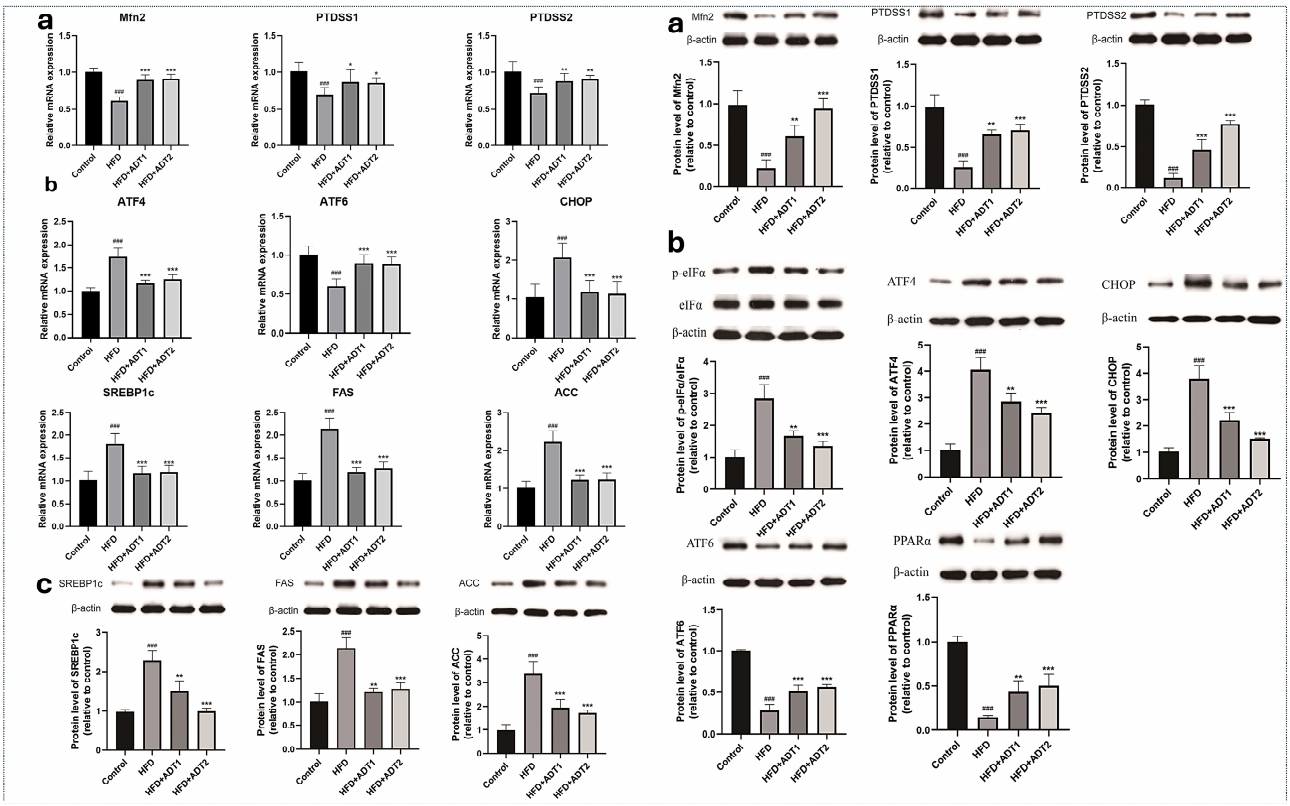
Data are expressed as mean±SD , ### P<0.001 versus Control group; *** P<0.001 versus HFD group.

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

Concurrently, a moderate yet statistically significant decrease is observed in the level of PC (P<0.05). However, when the mice were treated with ADT, there was a marked and statistically significant increase in the levels of PS, PE and PC. Furthermore, HFD significantly decreased the expression of Mfn2, PTDSS1 and PTDSS2 in the liver (shown in Fig. 6a), which are related to phospholipid synthesis and transformation. However, ADT could reverse this trend compared to the HFD group, as demonstrated in Fig. 6a.

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. 6b. In contrast to the control group, 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



A: Relative mRNA levels; B:Relative protein levels (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)

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), as demonstrated in Fig. 6b.

DISCUSSION

In our study, we have confirmed that Mfn2 is a critical protein involved in both the onset 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 therapeutic potential for NAFLD treatment involves the regulation of Mfn2 and PS 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, there is an insufficient import of PS into the mitochondria for its subsequent decarboxylation into 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. Our findings revealed 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 within the endoplasmic reticulum membrane are effective activators of the unfolded protein response (UPR). The UPR, in turn, modulates gene expression to counteract endoplasmic reticulum stress (ERS), leading to activation of eIF2α kinase and protein 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 p-ERK/eIF2α, ATF4 and CHOP, while ADT significantly decreased these elevated proteins. Acting as a

transcription factor of the unfolded protein, ATF6 can enhance fatty acid metabolism and proteostasis (Glembotski *et al.*, 2020). Over-expression of ATF6 also boosts the transcriptional activity of PPAR α . PPAR α can enhance fatty acid β -oxidation and oxidative phosphorylation.(Yang *et al.*, 2021). The transcription factor SREBP1c, responsible for initiating the expression of downstream genes that play a role in lipid biosynthesis, including ACC and FAS, can be suppressed by PPAR α , which in turn promotes the expression of the Insig2a gene· (Nguyen *et al.*, 2021). It is widely accepted that a 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 are paramount in modulating the function of hepatic cells dependent on the membrane, influencing various cellular attributes, including antioxidant activity, anti-inflammatory response, anti-fibrotic effects and cellular signaling pathways (Hernández-Alvarez *et al.*, 2019). A deeper understanding of these protein-lipid interactions provides insight into the fundamental mechanisms of NAFLD and enables the exploration of potential therapeutic targets and intervention 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. Finally, our research proposes a novel perspective on the utilization of H2S prodrugs, which could signify a new category of medications 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 H_2S. 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 H_2S.Based on existing literature, it is reasonable to speculate that H_2S may have an anti-fibrotic effect due to its ability to inhibit inflammatory factors. Chronic inflammation is a key contributor to fibrotic progression and studies have indicated H_2S exhibits potent 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 H_2S.

CONCLUSIONS

In conclusion, the study provides significant revelations regarding the part played by Mfn2 in both the onset and escalation 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 PS 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 research findings enrich the expanding understanding of the pathogenesis of NAFLD and offer new possibilities for therapeutic interventions. Further investigation is warranted to thoroughly understand the mechanisms by which Mfn2 and ADT influence NAFLD, as well as to assess the viability of H_2S as a therapeutic intervention for NAFLD treatment.

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