Metadoxine Prevents Endoplasmic Reticulum Stress and Apoptosis in Mice and Patients with Non-Alcoholic Steatohepatitis

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1. Abstract

Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver disorder. Although metadoxine successfully affects alcohol-associated steatohepatitis, its impact on non-alcoholic steatohepatitis (NASH) is mostly unknown. This study aims to explore the underlying mechanism of metadoxine and determine its impact and healing potential in NASH. We used and randomly divided male C57BL/6J mice into three groups of six animals: 1) a control group fed with standard diet; 2) a NASH group that received a high-fat diet (HFD); 3) a metadoxine group that received HFD and metadoxine (200 mg/kg). Patients with NASH were enrolled and assigned to receive metadoxine. Our results suggest that the mice developed severe hepatic steatosis and necroinflammation, accompanied by elevated serum aminotransferase and triglyceride, after 16 weeks of HFD. Metadoxine administration effectively reduced hepatic steatosis and inflammation; it also downregulated hepatic lipogenesis-associated and inflammation-related genes, and inhibited the induction of ER stress and apoptotic genes in HFD feeding mice. Consistently with the above major findings in mice, NASH patients treated with metadoxine achieved significant improvement in NASH. These findings revealed that Metadoxine can alleviate necroinflammation throughout reducing hepatic fat accumulation, ER stress, and cell apoptosis.

2. Introduction

Non-alcoholic fatty liver disease (NAFLD) represents the most typical chronic liver disease in the world. The overall prevalence of NA-FLD is around 25.24% worldwide, and the Middle East and South America were the most affected areas in 2016, while Africa showed the lowest prevalence [1]. Characterized by aberrant lipid deposition in hepatocytes, NAFLD comprises a broad spectrum of liver pathologies ranging between simple steatosis and non-alcoholic steatohepatitis (NASH) with or without fibrosis [2]. However, NASH can induce fibrosis, leading to cirrhosis and even NASH-related hepatocellular carcinoma [3]. Therefore, it is essential to explore the diagnosis and timely treatment of NASH.

Currently, no treatment has been approved for NASH by the US Food and Drug Administration or the European Medicines Agency. Although metformin, an insulin sensitizer, can improve serum aminotransferase and insulin resistance, it has no significant effect on liver histology [4, 5]. In contrast, the antioxidant Vitamin E could improve hepatic histology in adult individuals with biopsy-proven NASH [6]. Previously, statins were considered for NASH patients with hyperlipidemia, but this treatment likely poses a risk of hepatotoxicity [7]. Therefore, more clinical trials are required to provide efficient and safe drugs to several heterogeneous cases.

Metadoxine (pyridoxine-L-2-pyrrolidone-5-carboxylate) exerts multifaceted pharmacological effects appropriate for use in alcoholic liver disease [8], including the restoration of nicotinamide adenine dinucleotide (NADH), glutathione (GSH), and adenosine triphosphate level; the reinstatement of the balance of hepatic saturated/unsaturated fatty acids and esters; and the reduction of oxidative

stress [9]. In addition, metadoxine can effectively preserve the GSH reservoir and prevent lipid peroxidation, collagen disposition, and tumor necrosis factor-alpha secretion caused by ethanol and acetaldehyde in cultured liver cells [10]. Furthermore, previous studies have demonstrated that metadoxine is a potential therapy for NASH and NAFLD [11]. Therefore, the present work aimed to assess the effects and mechanisms of metadoxine on high-fat diet-induced steatohepatitis in mice.

3. Materials and Methods

3.1. Animals

Male C57BL/6J mice were obtained from Beijing HFK Bioscience Co., Ltd., at eight weeks of age. After adaptation for one week, the animals were randomized into three groups (n=6/group): Control (fed standard diet), NASH (fed high-fat diet (HFD) [TD.88137, Harlan Teklad] comprising 42%, 42.7%, and 15.2% calories from fats, carbohydrates, and proteins, respectively, at will for 16 weeks), and metadoxine groups (200 mg/kg/d metadoxine [QIDU Pharmaceutical, Shangdong, China] was added to HFD fodder for 16 weeks). Animal housing was carried out at 25 ± 3°C under a 12 h/12 h light-dark cycle. Bodyweight was recorded for every animal each week. At the end of the experiment, the mice were fasted for 8 hours and then anesthetized using isoflurane in chambers. The blood was collected from the eyeball veins of the mice; then, the liver tissues were collected and fixed in formalin or stored at -80°C until use. Animal housing, handling, and treatments were executed according to the animal handling protocol approved by the institutional ethics committee of the Third Hospital of Hebei Medical University.

3.2. Morphological Analysis of the Liver

The livers were removed rapidly, weighed, and dissected for histological examination. A portion of the liver samples underwent snap-freezing in liquid nitrogen and was kept at -80°C for subsequent analyses or Tissue-Tek OCT (Sakura) for Oil Red O staining. After paraffin embedding, liver samples underwent hematoxylin and eosin (H&E) staining according to standard protocols. In addition, abdominal fat mass was measured.

3.3. Biochemical Assays

Blood samples were collected after 8 hours of fasting. After centrifugation (10 min, 500 g; at 4°C) within 30 min of collection, total serum cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) amounts were assessed with commercially available kits on an autoanalyzer in the Clinical Chemistry Laboratory of Third Hospital of Hebei Medical University. Liver tissue with isopropyl alcohol was grounded thoroughly to 10% homogenate. The concentration of TC and TG in the livers was measured after evaporation of the organic solvent using a total cholesterol assay kit and triglyceride assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

3.4. Oil Red O Staining

Freshly collected liver tissue samples were embedded in Tissue-Tek 4583 OCT compound (Sakura Finetek, USA), sectioned at 8 µm, and submitted to Oil Red O staining. After fixation with 10% formalin (10 min), treatment with isopropanol (5 min), and stained with freshly prepared 60% Oil Red O working solution (7 min), the sections were treated with 85% isopropanol (3 min) and submitted to hematoxylin counterstaining (2 min).

3.5. Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA isolation from liver tissue samples was performed with TRIzol Reagent (Invitrogen) according to the manufacturer. Total RNA was assessed for purity on a NanoDrop system. Reverse transcription was performed from 1 µg of total RNA with a cDNA synthesis Kit (Life Technologies, USA). The amplification was carried out as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s. The primers are described in Table 1. Each experiment was carried out in triplicate. β-actin served as a housekeeping gene, and data were assessed by the $2^{-\Delta\Delta CT}$ method.

Table 1: Primers for quantitative qRT-PCR analysis.

Gene	Length of amplicon (bp)	Primers
PPARα	76	F 5′-TGCAGCCTCAGCCAAGTTGAA-3′ R 5′-TCCCGAACTTGACCAGCCA-3′
PPARγ	84	F 5′-GACGCGGAAGAAGAGACCTG-3′ R 5′-TCACCTTGTCGTCACACTCG-3′
SREBP1c	134	F 5′-CAGACTCACTGCTGCTGACA-3′ R 5′-GATGGTCCCTCCACTCACCA-3′
FASN	120	F 5′-CAAGTGTCCACCAACAAGCG-3′ R 5′-GGAGCGCAGGATAGACTCAC-3′
Acaca	198	F 5′-ACCAGTCACACCTACCCCATT-3′ R 5′-CACTGCTGGGCAACTCCTCA-3′
TNF-α	148	F 5′-CCTGTAGCCCACGTCGTAG-3′ R 5′-GGGAGTAGACAAGGTACAACCC-3′
IL-1β	116	F 5′-GAAATGCCACCTTTTGACAGTG-3′ R 5′-TGGATGCTCTCATCAGGACAG-3′
IL-6	199	F 5′-TTCCATCCAGTTGCCTTCTT-3′ R 5′-CAGAATTGCCATTGCACAAC-3′
IκBα	111	F 5′-AGGCTTCTGGGCCTTATGTG-3′ R 5′-TGCTTCTCTCGCCAGGAATAC-3′
NFκB	146	F 5′-TGAAGGACGAGGAGTACGAGC-3′ R 5′-TTCGTGGATGATTGCCAAGTG-3′
NRF2	108	F 5′-TGAAGCTCAGCTCGCATTGA-3′ R 5′-TGCTCCAGCTCGACAATGTT-3′
GRP78	78	F5′-TGTGTTGTGAGACCAGAACCG-3′ R 5′-GCAGTCAGGCAGGAGTCTTA-3′
PERK	84	F5′-AGAGGGCACCCACAAAACTT-3′ R 5′-GTCATCCCAACACACGCTCA-3′
eIF2α	174	F5′-TCCTCGTTGCCACTAAGCAG-3′ R 5′-AACAAGCTGACATAGGCCCC-3′
IRE1α	103	F5′-TGTGGTCAAGATGGACTGGC-3′ R5′-TCGGAGGAGGTCTCTCACAG-3′
AIF	192	F5′-AGGAGTGATCGCCGAAATGT-3′ R5′-CTGCCATCTTTCCCAGAAGC-3′
Caspase3	118	F5′-GAGCTTGGAACGGTACGCTA-3 R 5′-GAGTCCACTGACTTGCTCCC-3
Caspase8	106	F 5′-AAGCAGGAAGTGTGAGAGGC-3 R 5′-GATCCTCAGGAGGCACCTTG-3
Caspase9	156	F 5′-CACCTTCCCCAGGTTGCCAAT-3 R 5′-CAAGCCATGAGAGCTTCGGA-3
GAPDH	112	F5′-CAAGAAGGTGGTGAAGCAGG-3′ R5′-AAAGGTGGAGGAGTGGGTGT-3′

3.6. Western blot analysis

According to the manufacturer, total protein extraction was performed in a homogenizing buffer (RIPA) with protease inhibitors. The homogenates were then centrifuged at 4°C, and the resulting supernatants were collected. Total protein amounts were assessed by the BCA method. Equal amounts of total protein were resolved by SDS-polyacrylamide gel electrophoresis and electro-transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, UK). After blocking by incubation with 5% skim milk in TBST, the membranes underwent successive incubations with primary antibodies against peroxisome proliferator-activated receptor α/γ (PPARα/γ), sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FASN), phosphorylated acetyl-CoA carboxylase (p-Acaca), nuclear factor kappa-B (NF-κB) (ProteinTech Group, Chicago, IL, USA), tumor necrosis factor-α (TNF-α), interleukin 1 beta (IL-1β) (Abcam, Cambridgeshire, UK), interleukin 6 (IL-6), an inhibitor of NF-κB α (IκBα), p-IκBα, inositol-requiring enzyme-1α (IREα), glucose-regulated protein 78 (GRP78) (Santa Cruz Biotechnology, Santa Cruz, CA), PKR-like endoplasmic reticulum kinase (PERK), eukaryotic initiation factor 2α (eIF2α), p-PERK and p-eIF2α, apoptosis-inducing factor (AIF) (Cell Signaling Technology, Danvers, MA), cleaved caspase 3, 8, and 9 (Novus Biologicals, Littleton, CO, USA), and HRP-linked secondary antibodies (ProteinTech Group, Chicago, IL, USA). Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate Kit (Thermo Scientific, USA) was employed for detection, followed by densitometric quantitation with Image J (NIH). β-actin was used for normalization.

3.7. Measurement of ER stress and apoptosis in the liver tissue of NASH patients

A total of 16 NASH patients treated with metadoxine (1 g/d metadoxine [QIDU Pharmaceutical, Shangdong, China]) were enrolled from the Third Hospital of Hebei Medical University (Shijiazhuang, Hebei, China) in this study. Liver biopsy and pathological examinations were undertaken at baseline and 48 weeks of treatment. All liver biopsy specimens were processed for H&E staining and IHC staining of GRP78 (1:80; Affinity, Changzhou, China), p-PERK (1:100; Abways, Shanghai, China), and cleaved-caspase3 (1:100; Abways, Shanghai, China); and Tunel staining (Zhongshanjinqiao, Beijing, China). In addition, histological staging of the liver fibrosis was carried out according to NAS scores by three specialists.

3.8. Statistical Analysis

Data were expressed as mean ± standard deviation (SD) of the mean. One-way ANOVA was performed for group comparison. $P<0.05$ indicated statistical significance.

4. Results

4.1. Effects of metadoxine on body and liver weights and fat mass in HFD-fed mice

HFD-fed mice had uniformly pale fatty livers and hepatomegaly; metadoxine alleviated these conditions (Figure 1A). To determine whether metadoxine regulates steatosis, we measured body, liver weights, and fat mass. Body and liver weights besides abdominal fat mass markedly elevated in the HFD group (fed for 16 weeks) compared with control animals (Figure 1C, D). However, after treatment with metadoxine, these values were decreased compared to those of the HFD group (Figure 1C, D).

4.2. Effects of metadoxine on steatohepatitis in the HFD-induced NASH model

Hematoxylin-eosin staining of liver samples showed remarkable macro-vesicular and micro-vesicular steatosis in HFD mice with elevated multifocal necrosis compared to control animals. Meanwhile, metadoxine administration alleviated hepatic lipid disposition and decreased focal necroinflammation (Figure 1B). Consistently, Oil Red O-staining showed elevated amounts of lipid droplets in HFD-fed animals compared with controls; metadoxine also alleviated this effect (Figure 1B). The NAFLD activity scores (NAS) were 0.5 ± 0.7, 5.8 ± 0.8, and 2.2 ± 0.8 in the controls, HFD-fed mice, and metadoxine administrated mice, respectively (Figure 1F); the differences among the three groups were significant ($P = 0.000$). In addition, the NAS scores were remarkably higher in HFD-diet mice than in the livers of control mice ($P = 0.000$), which were significantly ameliorated by metadoxine treatment ($P = 0.006$, Figure 1F). The above findings indicate that metadoxine efficiently improved high-fat diet-associated steatohepatitis.

4.3. Metadoxine improves serum biochemistry and hepatic lipid disposition in HFD-induced NASH mice

In comparison with control animals, the HFD group had remarkably increased serum alanine transaminase (ALT), aspartate transaminase (AST) (Figure 1G, H), liver triglyceride (TC), and low-density lipoprotein (LDL)-C levels (Figure 1I, K). Metadoxine-treated mice showed reduced serum ALT, AST, TC, and LDL-C amounts compared with HFD-fed mice (Figure 1G, H, I, K). The concentrations of hepatic TC and TG in the HFD-fed mice were significantly higher than those in the control mice (TC, 1.14 ± 0.09 vs 0.45 ± 0.07 mmol/L, $P = 0.000$; TG, 5.86 ± 0.46 vs 4.64 ± 0.06 mmol/L, $P = 0.01$, Figure 1L, M). Metadoxine administration was able to alleviate HFD-induced hepatic lipid accumulation, especially triglyceride (4.93 ± 0.13 mmol/L, $P = 0.028$) and necroinflammation.
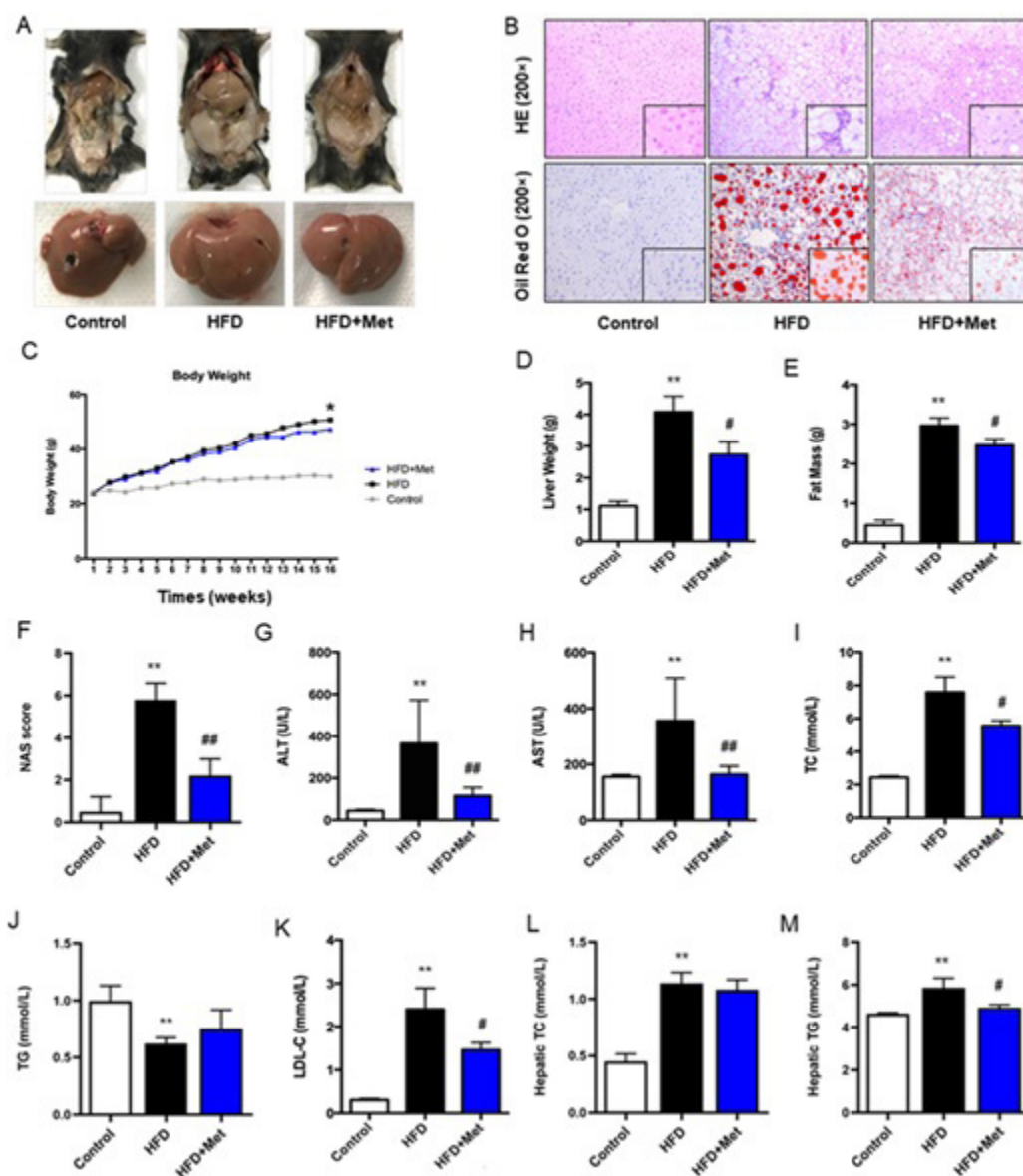
Figure 1: Metadoxine protects against dietary-induced steatohepatitis and liver injury. (A) Representative images of livers from mice. (B) Histopathological changes of the liver sections from mice fed with control or HFD. Hematoxylin and eosin staining (up, original magnification ×200) and oil red O staining (down, original magnification ×200). (C) Bodyweight curve in each group. (D and E) Liver weight and fat mass in Control, HFD, and metadoxine-treated mice. (F) NAS score in each group. (G, H, I, J, and K) Levels of ALT, AST, TC, TG, and LDL-C in the plasma in the control, HFD, and metadoxine-treated mice. (L and M) Levels of hepatic TC and TG in liver tissue in three groups. Values are mean ± SD, **$P < 0.01$ compared with control, #$P < 0.05$, ##$P < 0.01$ compared with HFD.

4.4. Effects of metadoxine on hepatic lipid metabolism in HFD-fed mice

To explore metadoxine's underlying mechanisms on steatosis, we assessed lipogenic markers by qPCR and Western blot. Genes that contribute to fatty acid and triglyceride syntheses, e.g., *PPARa/γ*, were significantly decreased. At the same time, SREBP1c and the responsive molecules FASN and p-Acaca showed increased amounts in HFD-fed mice at both mRNA and protein levels (Figure 2A, B). Moreover, metadoxine administration reversed these effects (Figure 2A, B). These results indicate that metadoxine might alleviate lipid metabolism disorders in HFD-induced steatohepatitis.

4.5. Effects of metadoxine on hepatic inflammation in HFD-fed mice

To explore metadoxine's effect in preventing hepatic inflammation, we determined the expressions of the proinflammatory molecule by qPCR and western blot. The expressions of TNFα, IL-1β, IL-6, NF-ϰB, and IϰB mRNAs and protein significantly increased in HFD-fed mice compared to the control mice. Thus, oral supplementation of metadoxine could suppress the release of the inflammatory factors (Figure 2C, D). Thus, metadoxine might ameliorate steatohepatitis by decreasing the expression of hepatic pro-inflammation cytokines.
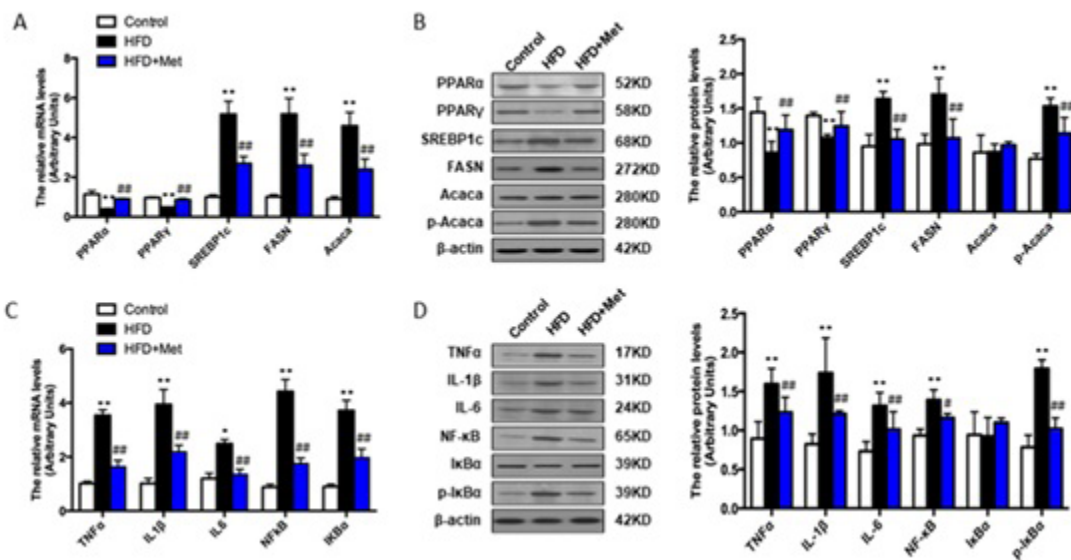
Figure 2: Metadoxine inhibits hepatic lipid metabolism-related and inflammation-related gene and protein expression in HFD-induced steatohepatitis mice. (A) mRNA levels of the lipid metabolism-related genes (PPARα, PPARγ, SREBP1c, FASN, Acaca) in livers from mice. (B) Western blot for the indicated lipogenic genes (PPARα, PPARγ, SREBP1c, FASN, Acaca, p-Acaca). β-actin was used as a loading control. (C) mRNA levels of the inflammation-related genes (TNFα, IL-1β, IL-6, NF-κB, IκBα) in livers from mice. (D) Western blot for the inflammation-related proteins (TNFα, IL-1β, IL-6, NF-κB, IκBα, and p-IκBα). β-actin was used as a loading control. Values are mean ± SD, *P < 0.05, **P < 0.01 compared with control; #P < 0.05, ##P < 0.01 compared with HFD.

4.6. Metadoxine inhibits the ER stress signaling pathway in NASH animal models

To assess a potential association of metadoxine with hepatic ER stress, GRP78 and IRE1α amounts were assessed as essential molecules in ER stress and lipid biogenesis. The mRNA levels of *GRP78* and *IRE1a* were intensely augmented in the HFD group and were reversed by the metadoxine treatment (Figure 3A). The mRNA and protein amounts of PERK and eIF2α were comparable in both groups (Figure 3A, B). However, the protein levels of GRP78, p-PERK, p-eIF2α, and p-IRE1α protein levels strongly increased, and the administration of metadoxine abolished these effects (Figure 3B, C). These results suggested that metadoxine can ameliorate NASH by diminishing the ER stress signaling pathway.
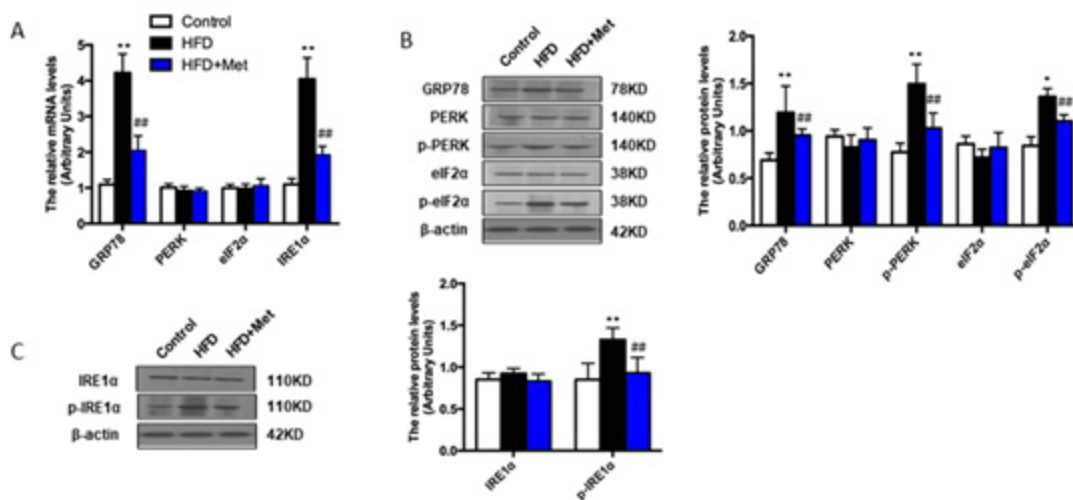

Figure 3: Metadoxine inhibits ER stress-related genes and proteins expression in HFD-induced steatohepatitis mice. (A) mRNA levels of the ER stress-related genes (GRP78, PERK, eIF2α, IRE1α) in livers from mice. (B) The expression levels of ER stress-related protein (GRP78, PERK, p-PERK, eIF2α, p-eIF2α) were confirmed by western blot. (C) The protein levels of IRE1α and p-IRE1α were evaluated by western blot. β-actin was used as a loading control. Values are mean ± SD, *P < 0.05, **P < 0.01 compared with control; ##P < 0.01 compared with HFD.

4.7. Metadoxine inhibits apoptosis in HFD-induced steatohepatitis

Next, we assessed whether critical apoptotic genes were closely related to NASH. Interestingly, HFD markedly upregulated apoptosis-inducing factor (AIF) and cleaved caspase 3, 8, and 9 at both mRNA and protein levels (Figure 4). Meanwhile, the administration of metadoxine suppressed apoptosis by decreased AIF and cleaved-caspase 3, 8, and 9 amounts compared with the HFD group (Figure 4A, B), indicating that metadoxine might exert anti-apoptotic effects in HFD-induced steatohepatitis.

4.8. Metadoxine treatment alleviates steatosis and inflammation in patients with NASH

With the interesting findings in mice, we further examined the effects of metadoxine on NASH and potential molecular mechanisms in a human study. A total of 16 NASH patients underwent paired liver biopsies at baseline and after 48 weeks of treatment. HE staining showed that hepatic lipid disposition and focal necroinflammation were improved after treatment of metadoxine (Figure 5A). However, NAS scores were decreased in 9/16 patients (56.3%) after metadoxine treatment (Figure 5B).

4.9. Effects of metadoxine treatment on ER stress and apoptosis pathway in NASH patients

IHC results for GRP78, p-PERK, and cleaved-caspase3, and a Tunel staining in the liver tissues of NASH patients at baseline and 48 weeks of metadoxine treatment were showed in Figure 5A. Notably, metadoxine exhibited significantly greater effects on the decreases in GRP78 and p-PERK expression ($P<0.001$, Figure 5C, D). In addition, the IHC assay of cleaved-caspase3 and Tunel staining showed that metadoxine reduced apoptosis in patients with NASH ($P<0.05$, Figure 5E, F).
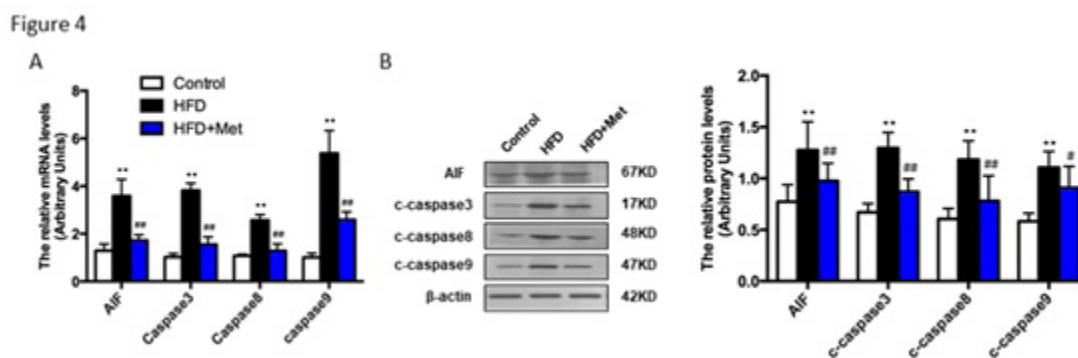

Figure 4: Metadoxine suppresses apoptosis in HFD-induced steatohepatitis. (A) The mRNA levels of hepatic apoptosis genes (AIF, caspase3, caspase8, caspase9) were performed by qPCR. (B) Western blot for the apoptosis-related protein (AIF, c-caspase3, c-caspase8, and c-caspase9). β-actin was used as a loading control. Values are mean ± SD, **P < 0.01 compared with control.
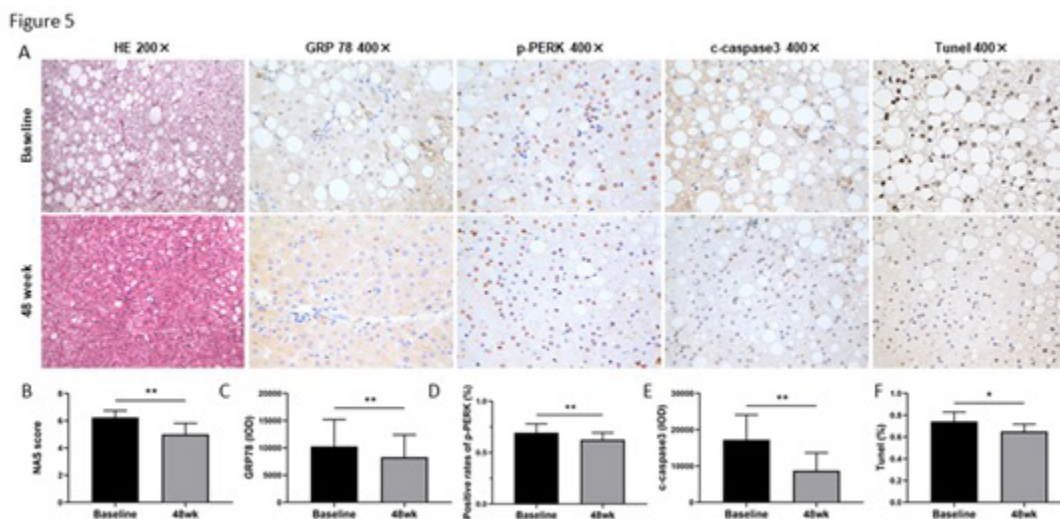

Figure 5: HE staining, Tunel staining, and IHC of hepatic GRP78, p-PERK, c-caspase3 from NASH patients treated with metadoxine. (A) Representative images of HE staining, Tunel staining, and IHC of hepatic GRP78, p-PERK, and c-caspase3 from NASH patients treated with metadoxine at baseline (upper panels) and after 48-week treatment (lower panels) (original magnification ×200). (B) The decrease of NAS score after 48-week treatment of metadoxine. (C) The decrease of GRP78 expression after 48-week treatment of metadoxine. (D) The reduction of p-PERK positive cells after 48-week treatment of metadoxine. (E) The decrease of c-caspase3 expression after 48-week treatment of metadoxine. (F) The reduction of apoptosis cells after 48-week treatment of metadoxine.

5. Discussion

In this paper, we used animal models and NASH patients to assess the protective effect of metadoxine in steatohepatitis to provide further insights into the pharmacology of metadoxine in NASH treatment. The experiment focused on studying the underlying mechanisms of metadoxine treatment in NAFLD, using an HFD-associated steatohepatitis mouse model. Additionally, the efficacy of metadoxine in the NASH animal model was evaluated. We found that metadoxine could alleviate liver injury during NASH progression since ALT and AST levels significantly decreased in metadoxine-treated mice compared to HFD-fed mice.

Multiple mechanisms contribute to NASH pathogenesis, particularly lipid metabolism disorders [12]. The results shown in liver sections submitted to Oil Red O staining demonstrated that metadoxine treatment significantly inhibited lipid accumulation; the lipid metabolism disorders observed in HFD-associated steatosis involve PPARα and PPARγ downregulation. As expected, metadoxine abolished these effects. PPARs belong to the superfamily of nuclear receptors, fatty acid-activation transcription factors [13, 14]. As demonstrated in our previous studies, PPARs regulate hepatic lipid metabolism and carry critical roles in NAFLD [15–17]. In metadoxine-treated mice, the reduction of HFD-associated elevation of serum TC, LDL-C was consistent with the decrease of hepatic lipid disposition. Consequently, metadoxine may alleviate steatohepatitis via modulation of fatty acid oxidation, and also involved PPARα and PPARγ activation.

As shown above, the gene and protein levels of molecules regulating SREBP1c, an essential modulator of *de novo* lipogenesis, and the downstream lipogenic proteins FASN and Acaca [18, 19] decreased following the administration of metadoxine. The liver fat deposition markedly decreased upon metadoxine treatment. As such, we can attribute this outcome to the concurrent effects of metadoxine on lipogenic genes (*SREBP1c*, *FASN*, and *Acaca*). To conclude, the overall outcome of lipogenic gene changes suggests that metadoxine can reduce the utilization and storage of fatty energy resources in the liver, increasing their export and use by peripheral tissues. Excessive body fat will disturb lipid metabolism and cause the concordance of metabolism and immune response, which induces chronic inflammatory status. As a transcription factor, NF-ϰB is involved in regulating the expression of various proinflammatory cytokines (TNF-α, and IL-1β, among others) during NASH development [20]. NF-ϰB in its resting state is located in the cytoplasm and is bound to its inhibitor of NF-ϰBα (IϰBα). Activation of IϰB kinase leads to the phosphorylation, dissociation, and subsequent degradation of the IϰBα protein; as a result, NF-ϰB is released. TNF-α, as an essential stimulant, can induce the degradation of IϰBα, thereby promoting the activation of NF-ϰB [21]. Thus, activation of NF-ϰB can aggravate oxidative stress and inflammatory responses and jointly accelerate NAFLD progression. Our research suggested that HFD stimulation markedly upregulated the expression of NF-ϰB, IL-1β, IL-6, TNF-α, and IϰB-α, which could be suppressed by metadoxine administration.

Elevated lipotoxic stress (increased saturated fatty acids) could induce the ER stress response [22, 23]. Fat deposition in hepatocytes is associated with increased hepatic ER stress [24, 25]. In the performed experiment, HFD-induced steatohepatitis increased GRP78 expression, and metadoxine administration during NASH progression decreased it. GRP78 (or Bip) is an essential ER chaperone critical for proper protein folding, degradation of misfolded proteins, and the induction of transmembrane ER stress sensors such as protein kinase RNA-activated (PKR)-like ER kinase (PERK) and inositol requiring enzyme (IRE) 1 [26]. GRP78 is also associated with insulin activity, fat metabolism-related inflammation, and cell death in NAFLD [27]. This study demonstrated that metadoxine could decrease HFD-induced upregulation of ER transmembrane signaling effectors, including PERK and IRE. Besides, hepatic ER stress plays an essential role in controlling the content and size of lipid droplets and regulating the biosynthesis of lipids via sterol regulatory element-binding proteins (SREBP) [28, 29]. SREBP1a and SREBP1c are isoforms of SREBP1 [30]. A recent study found that ER stress in *ob/ob* mice promotes SREBP-1c production and enhances liver lipogenesis [31]. This study disclosed a coordinated transcriptional suppression of genes associated with lipogenesis and ER stress, such as PERK, eIF2α, and IRE1α. These results may imply an inhibitory role for metadoxine in ER stress response during NASH to reduce the transcription of lipogenesis-associated genes and alleviate subsequent lipotoxic stress and injury.

Apoptosis affects NASH progression, abnormalities in lipid metabolism and reactive oxygen species synthesis, and ER stress accelerate apoptosis [32]. Hepatocytes undergoing apoptosis induce immune and hepatic stellate cells toward fibrosis progression via proinflammatory cytokines [33]. However, whether metadoxine protects against apoptosis-induced NASH remains unclear. In this study, metadoxine inhibited the high-fat diet-induced expression of AIF, a crucial regulator of caspase-independent apoptosis signaling. Moreover, metadoxine also inhibited the caspase-dependent apoptosis signaling pathway, as demonstrated by the suppression of cleaved caspase 3, cleaved caspase 8, and cleaved caspase 9 after metadoxine administration. Caspase 3 activation in NASH is highly associated with hepatocyte apoptosis and NASH progression [34]. Caspase 8 activation is an essential contributing factor in NASH [35]. Its deficiency in hepatocytes reduced MCD-induced apoptosis and downregulated proinflammatory cytokines [36].

We extended our findings in mice to a human study of patients with NASH, in which 16 patients underwent liver biopsy at baseline and after 48-week treatment. Notably, 56.3% of patients taking metadoxine achieved an improvement in NAS score. Furthermore, the hepatic GRP78 and p-PERK levels and apoptosis of hepatocytes in NASH patients were abrogated significantly by metadoxine treatment. Hence, metadoxine improves NASH through ER stress and apoptosis pathway in NASH patients.

6. Conclusion

The data collected indicate that oral supplementation of metadoxine protects mice and patients from nutritional hepatic steatosis and necroinflammation, which might be associated with suppression of hepatic lipogenesis endoplasmic reticulum stress, and apoptosis. Thus, the present findings provide a novel insight into the effects of metadoxine treatment of nutritional steatohepatitis.

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