Docosaehaenoic Acid Lessens Hepatic Lipid Accumulation and Inflammation via the AMP-Activated Protein Kinase and Endoplasmic Reticulum Stress Signaling Pathway in Grass Carp (*Ctenopharyngodon Idella*)

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Short report

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Abstract

Background: The liver is the primary organ for frontline immune defense and lipid metabolism. Excessive lipid accumulation in the liver severely affects its metabolic homeostasis and causes metabolic diseases. Docosahexaenoic acid (DHA) is known for its beneficial effects on lipid metabolism and anti-inflammation, but its molecular mechanism remains unknown, especially in fish. In this study, we evaluated the protective effects of DHA on hepatic steatosis of grass carp (*Ctenopharyngodon idella*) *in vivo* and *in vitro* and mainly focused on lipogenesis and inflammation. Grass carp were fed with purified diets supplemented with 0%, 0.5% and 1% DHA for 8 weeks *in vivo*. Hepatocytes were treated with palmitic acid (PA) (200 μ M) with or without DHA (50 or 100 μ M) for 24 h *in vitro*. In addition, Compound C (CC, the inhibitor of AMP-activated protein kinase) was used to examine the mechanism of DHA on hepatic steatosis *in vitro*.

Results: In this study, 1% DHA significantly decreased the liver triglyceride (TG), malondialdehyde (MDA), serum tumor necrosis factor α (TNF α) and nuclear factor kappa B (NF κ B) contents. DHA (100 μ M) effectively attenuated PA-induced lipid accumulation ($P < 0.05$). Furthermore, DHA significantly inhibited endoplasmic reticulum (ER) stress and stimulated the expression of AMP-activated protein kinase (AMPK) and its downstream factors related to hepatic inflammation and lipogenesis *in vivo* and *in vitro*. However, the effects of DHA could be abrogated by CC *in vitro*.

Conclusions: DHA exerted a protective effect on hepatic steatosis by inhibiting ER stress, improving antioxidant ability, relieving hepatic inflammation and inhibiting hepatic lipogenesis in an AMPK-dependent manner. Our findings give a theoretical foundation for further elucidation of the beneficial role of DHA in vertebrates.

1. Background

Aquatic products are known as high-protein and low-fat food, which are popular among different people worldwide. Therefore, the production of aquatic products develops the aquaculture industry and provides economic benefits. A high-energy diet is frequently used in aquaculture to increase growth speed and shortens the fish production cycle. Prolonged energy imbalance is associated with ectopic fat deposition, mainly in the liver and viscera [1, 2]. It promotes the prevalence of metabolic syndrome diseases (e.g., hepatic steatosis and liver inflammation) in aquatic animals [3, 4]. Thus, it is important to explore mechanisms exerting liver protection in the aquaculture industry.

Hepatic steatosis is one of the most common chronic liver diseases in humans, which is mainly induced by high-energy diets [5]. Fatty liver can cause hepatitis, fibrosis and irreversible end-stage liver diseases, such as liver cirrhosis and cancer [6]. Fat overload is the critical predisposing factor for hepatic steatosis [7, 8]. Different studies have focused on lipid accumulation in response to other nutrients and key molecules causing hepatic steatosis [9–11]. Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) are known as n-3 highly unsaturated fatty acids (HUFAs). HUFAs promote various

critical physiological roles, such as growth performance improvement, cell membrane fluidity control, lipid metabolism regulation and immune function improvement in fish [12–14]. Studies have outlined numerous positive biological DHA impacts, such as anti-fibrogenic, anti-inflammatory and anti-obesity effects on liver protection [15, 16]. Compared with the other n-3 fatty acids in the liver, DHA shows potent anti-fibrogenic effects by interfering with pro-inflammation transforming growth factor- β (TGF β) and nuclear factor kappa-B (NF- κ B) (signaling pathways [15]. However, DHA shows anti-inflammatory effects in several ways under different conditions. These include up-regulation of peroxisome proliferator-activated nuclear receptor- γ (PPAR γ), prevention of macrophage infiltration, activation of NF- κ B and Jun N-terminal Kinase (JNK) signaling pathways, suppression of leukocyte chemotaxis and blocking of pro-inflammatory cytokine and eicosanoid production [17–19]. Furthermore, data from mice fed a high-fat diet showed that DHA could down-regulate G Protein-Coupled Receptor 120 (GPR120) and stimulate the browning of white adipose tissue [17, 20, 21]. Although there are some potentially beneficial biological effects of DHA, the molecular mechanism by which DHA exerts its nutritional effects in the liver was not detected in fish.

DHA ameliorates the endoplasmic reticulum (ER) stress and lipid accumulation in hepatocytes. Hepatocytes contain numerous ER, which play an essential role in hepatocyte homeostasis [22]. ER stress is one of the critical factors in the pathogenic mechanism of lipid-related metabolic diseases (e.g., hepatic steatosis) [23]. ER stress is characterized by the up-regulated expression of marker genes [C/EBP homologous protein (CHOP), glucose-regulated protein 78 (GRP78) and phosphorylated protein kinase-like ER kinase (p-PERK)] [24]. ER stress evokes hepatic steatosis through increasing lipogenesis or decreasing lipoprotein secretion [23]. Therefore, relieving ER stress may be a useful method for the therapy of hepatic steatosis.

Grass carp (*Ctenopharyngodon idella*) is the most abundant freshwater aquaculture fish in China. It deposits excess fat in adipose tissue and liver during farming. So, it is considered a valuable model for studying hepatic steatosis [25]. Previously, our studies observed that moderate dietary intake of n-3 HUFAs is beneficial to improve growth performance and physiological conditions of grass carp and alleviates hepatopancreatic lipid accumulation [14, 26]. We assumed that the protective effects of DHA on the liver could be associated with its ability to inhibit lipid accumulation. However, the exact molecular mechanism in fish remained obscure. Thus, this study aimed at investigating the underlying molecular mechanism of DHA that protects the liver.

2. Materials And Methods

Two experiments were performed *in vivo* and *in vitro*. All procedures were approved by the Northwest A&F University Institutional Animal Care and Use Committee.

2.1. Experiment 1: *in vivo* study

2.1.1. Experimental diets

Three isonitrogenous and isoenergetic purified diets were formulated by adding different concentrations of DHA (Net content of 50% Xunda Marine Biological Products Co., Ltd., Jiangsu, China). The fish were divided into three groups based on the concentrations of DHA, including Control (0% DHA), and 0.5% and 1% DHA groups. The formulation and proximate composition of the experimental diets are demonstrated in Table 1. Soybean oil (Kerry Oils & Grains Co., Shenzhen, China) and linseed oil (Hoval Seasons Bio-Sci Co., Changchun, China) were added to supplement 1% linoleic acid (LA) and 1% alpha-linolenic acid (LNA) essential fatty acid requirements, respectively. The experimental diets were prepared according to previously described methods [10].

2.1.2. Experimental procedure

Fish culture and management procedures used in this study were described by Shi et al. [25]. A total of 108 juvenile grass carp (initial weight: 29.76 ± 2.34 g) were distributed into 9 tanks (225-L) with 12 fish per tank. Diets were randomly assigned to three replicate tanks, and the feeding experiment lasted for 8 weeks.

At the end of the experiment, the fish were euthanized via immersion in tricaine methanesulfonate (MS-222; 100 mg/L) (Sigma, St. Louis, MO, USA). The final body weight (FBW, g), body length (BL, cm), relative intestine length (RIL, %), hepatosomatic index (HSI, %), condition factor (CF, g/cm³), survival rate (SR, %), viscerosomatic index (VSI, %), kidney index (KI, %) and spleen index (SI, %) of each fish were determined. Subsequently, triglyceride (TG) content, hematoxylin & eosin (H&E) staining, fatty acid composition, antioxidant enzymatic activities and relative gene expression levels of lipid synthesis, inflammation and ER stress were analyzed in the liver tissues.

An automatic biochemical analyzer (Hitachi 7180, Tokyo, Japan) was used to examine the serum biochemical indices, including alanine aminotransferase (ALT, U/mL), aspartate aminotransferase (AST, U/mL), total protein (TP, g/L), albumin (ALB, g/L), globulin (GLB, g/L), albumin and globulin (A/G) ratio, high-density lipoprotein cholesterol (HDL-c, mmol/L) and low-density lipoprotein cholesterol (LDL-c, mmol/L).

2.2. Experiment 2: in vitro study

2.2.1. Cell culture and treatment

We cultured the grass carp hepatocyte L8824 cell line as described previously [27]. The palmitic acid (PA; article no.: P5585) and DHA (article no.: D2534) were obtained from Sigma Aldrich (St. Louis, Missouri, USA). PA and DHA were dissolved in ethanol (Zhiyuan Chemical Reagent Co., Ltd., Tianjin, China) to prepare 100 mM stock solution and then diluted with a medium containing 2% free fatty acid (FFA)-free bovine serum albumin (BSA) to obtain the designed concentrations before use. PA (200 μ M) with or without DHA (50 or 100 μ M) was used to treat hepatocytes for 24 h. We added 10 μ M Compound C (CC; an AMPK inhibitor, HY-13418; Medchem Express Inc., Monmouth Junction, NJ, USA) in water, and the AMPK inhibitor was added 2 h before fatty acid treatment. Briefly, the experiment was divided into four

experimental groups: Control, 200 μ M PA, 200 μ M PA +DHA (50 or 100 μ M) and 200 μ M PA +100 μ M DHA+10 μ M CC (pretreatment for 2 h).

2.3. Sample analysis

2.3.1. Fatty acid composition analysis in experimental diets and liver samples

We used gas chromatography (GC) to detect fatty acid composition as described previously [25]. The fatty acid composition of the experimental diets is observed in Table 3.

2.3.2. TG content, malondialdehyde (MDA) content and enzymatic activity assays on the liver and hepatocytes

The TG content was measured using a tissue triglyceride assay kit (Applygen Technologies Inc., Beijing, China). Then, antioxidant enzymatic activities, such as superoxide dismutase (SOD) and catalase (CAT), were measured [25]. The end products of lipid peroxidation MDA were measured using assay kits (Jian Cheng Biochemical Co. Nanjing, China).

2.3.3. Liver histological processing and morphological evaluation, cell viability assays and Nile red staining

We prepared liver sample sections and stained them with H&E, as described previously [28]. These histological samples were observed and photographed under upright microscopy (Leica Biosystems, Germany), and relative cell viability was determined following standard instructions of the Cell Counting Kit-8 (K009-100T, Zeta Life Inc., USA) [29]. Briefly, hepatocytes were plated in a 96-well plate (1×10^4 cells/well) and cultured until these reached 85-90% confluence. Then, hepatocytes were treated with different concentrations of PA (100 or 200 μ M), DHA (50 or 100 μ M) and 200 μ M PA +100 μ M DHA for 24 h. Then, we added 10 μ L CCK-8 solution to each well (total reaction volume of 110 μ L) and incubated for another 3 h. The absorbance was detected at the wavelength of 450 nm. For observing the effect on lipid deposition, Nile red staining was used to view the lipid droplets in hepatocytes [30].

2.3.4. Investigation of the concentrations of pro-inflammatory cytokines in serum by ELISA assay

Pro-inflammatory cytokine concentrations, including TNF α and NF κ B, were examined using Fish ELISA Assay kits (FANKEL Industrial Co., Ltd, Shanghai, China).

2.3.5. Transcriptomic analysis of the liver samples

We detected the transcriptome of DHA-fed grass carp liver as described by Jing et al. [31]. Briefly, total liver RNA (2 fish/tank) was extracted, and its concentration, purity and integrity were determined. Subsequently, the total RNA from fish in each tank was mixed in equivalent amounts (3 replicates per treatment). RNA was enriched, fragmented and reverse transcribed into complementary DNA (cDNA), and the second cDNA strand was synthesized. Consequently, the cDNA fragments were purified using

magnetic bead precipitation and terminally repaired. poly (A) was added and attached to Illumina sequencing adapters. Finally, ligation products were size-selected through agarose gel electrophoresis, amplified using polymerase chain reaction (PCR) and sequenced using the Illumina novaseq 6000 platform (Sangon Biotech (Shanghai) Co., Ltd., China) following the manufacturer instructions.

2.3.6. Quantitative real-time PCR (qPCR) and western blot analysis using liver samples and hepatocytes

We investigated gene transcription levels based on our previous study [10]. The various primers used in this study are given in Table 2. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to calculate the target gene expression values [32]. We obtained protein translation levels from our previous study [10]. The primary antibodies against FAS (WL03376, 1:1000, Wanleibio) and β -Actin (3779, 1:1000, Prosci) were applied. Finally, the secondary antibody, goat anti-rabbit horseradish peroxidase conjugate (A02010, 1:2000, Abbkine), was added.

2.4. Statistical analysis

All statistical analyses were performed with SPSS 25.0 software (SPSS, Chicago, IL, USA). Percentage data, such as the fatty acid composition of the liver, were arcsine-transformed before analysis [33]. The Shapiro–Wilk test was done to analyze the normality of the data distribution, and Levene’s test was performed to examine the homogeneity of the variances. The differences among the treatments were analyzed by one-way analysis of variance (ANOVA) and compared by Duncan’s test. The student’s *t*-test was used for 2-group comparisons *in vivo* and *in vitro*. All data were then expressed as mean $\pm$ standard deviation (SD), and $P < 0.05$ was considered significant.

3. Results

Experiment 1: In vivo study

3.1. Growth performance and biological indices

In the 1% DHA group, FBW was higher than that in the other two groups (Table 4, $P \leq 0.05$). Also, compared with the control and 0.5% DHA groups, it effectively increased BL and decreased RIL and VSI, respectively (Table 4, $P \leq 0.05$). However, no significant differences in CF, SR, KI and SI were observed among the three treatments (Table 4). Serum ALT, ALB, HDL-c and LDL-c levels were altered in the 1% DHA group than those in the control group (Table 5, $P < 0.05$). As illustrated in Table 5, AST, TP, GLO and A/G showed no significant differences among the three treatments.

3.2. Fatty acid composition of liver

In the liver samples, the DHA proportion was increased in fish-fed DHA in a dose-dependent manner compared with the control group (Table 6, $P \leq 0.05$). Besides, the n-3HUFA, n-3PUFA, PUFA and n-3PUFA/n-6PUFA proportions were significantly increased in the 1% DHA group (Table 6, $P \leq 0.05$), whereas SFA was significantly decreased (Table 6, $P \leq 0.05$).

3.3. DHA attenuates lipid accumulation and improves the antioxidant capacity of the liver

We evaluated the effects of DHA on hepatic steatosis *in vivo*. Compared with the control group, histopathological examination based on H&E staining showed that the DHA-fed fish effectively reduced lipid vacuoles and significantly increased the HSI value (Figs. 1A and B, $P \leq 0.05$ except 0.5% DHA group). Liver TG contents were significantly reduced in the 1% DHA group compared with the control group (Fig. 1C, $P \leq 0.05$).

In the 1% DHA group, MDA contents were significantly reduced than those in the control group (Fig. 1D, $P \leq 0.05$). Although there were no significant differences in the SOD and CAT enzyme activities between the control and DHA groups, and there was an upward trend (Fig. 1E). Furthermore, the *SOD* and *CAT* mRNA assays confirmed the enzymatic activity assay results (Fig. 1F, $P \leq 0.05$).

3.4. DHA inhibits liver inflammation and hepatic lipid synthesis

NF κ B and TNF α serum levels were detected to clarify whether DHA had protective effects against inflammation *in vivo*. These parameters were significantly reduced in the 1% DHA group than those in the control group (Figs. 2A and B, $P \leq 0.05$). Additionally, this group showed a reduced expression in genes, including *TNF α* , *NF κ B*, *IL-1 β* , *IL-6* and *IL-8*, during liver inflammation compared with the control group (Figs. 2C and D, $P \leq 0.05$).

We assessed whether DHA would attenuate lipogenesis-related gene expression in the liver. *SREBP1c*, *C/EBP1 α* , *FAS* and *SCD1* mRNA expression levels were significantly reduced after adding 1% DHA (Figs. 2E and F, $P \leq 0.05$).

3.5. DHA activates AMPK and inhibits ER stress signaling pathways

In order to determine the underlying mechanism of DHA inhibiting hepatic steatosis and improving liver inflammation in grass carp, a functional enrichment analysis was done on the transcriptome data. The analysis showed that the lipid uptake (LDL-receptor) and metabolism and AMPK signaling pathways were up-regulated in the 1% DHA group compared with the control group (Figs. 3A and B).

Subsequently, we examined the *AMPK* expression in the mRNA level, which was increased nearly 1.5-fold in the 1% DHA group than the control group (Fig. 3C, $P \leq 0.05$). During ER stress, the mRNA expression levels of related genes, including *CHOP*, *GRP78*, *IRE1*, *ATF6* and *PERK*, were significantly decreased in the 1% DHA group (Figs. 3D and E, $P \leq 0.05$).

Experiment 2: In vitro study

3.6. PA-induced cell injury

In palmitate-treated cells, cytotoxicity occurred in a dose-dependent manner, and their cell viability was decreased by half when treated with PA (200 μ M) (Figs. 4A and E, $P \leq 0.05$). Besides, PA (200 μ M)

significantly increased the hepatocyte TG contents and up-regulated the hepatic lipid synthesis and liver inflammation-related-gene expression (Fig. 4B-D, $P \leq 0.05$).

We performed CCK8 analyses [29] to assess the cytotoxic effects of DHA, and determined the optimal concentration for hepatocyte experiments. Besides, 50 μM or 100 μM DHA did not display any toxicity in the cells, and co-treatment with 100 μM DHA prevented cytotoxic effects of PA (Fig. 4E, $P \leq 0.05$). Finally, cell viability indicated that the DHA supplementation significantly enhanced cellular cytotoxicity (Fig. 4E, $P \leq 0.05$).

3.7. DHA ameliorates PA-induced inflammation and lipid synthesis in hepatocytes

This study detected the TG contents and Nile red staining results to confirm whether DHA had a protective effect against palmitate-induced cell injury in grass carp hepatocytes. As illustrated in Fig. 5A, the intracellular neutral lipid Nile red staining demonstrated that DHA effectively alleviated PA-induced cellular damage. In the PA group, TG cell contents were increased than those in the control group, whereas DHA treatment significantly reduced PA-induced TG accumulation (Fig. 5B, $P \leq 0.05$). We examined whether DHA decreased the related gene expression during hepatic lipid synthesis and hepatocyte inflammation *in vitro*. DHA treatment significantly reduced the expression of the corresponding genes, including *FAS*, *SREBP1c*, *SCD1*, *C/EBP α* , *TNF α* , *IL-6*, *NF κ B* and *IL-1 β* (Figs. 5C and D, $P \leq 0.05$). Furthermore, in the PA group, the *FAS* expression levels were increased than those in the control group. In contrast, DHA supplementation restrained this trend (Fig. 5E).

3.8. DHA activates AMPK and inhibits ER stress signaling pathway to influence hepatic lipid synthesis and inflammation

In vivo, DHA supplementation activated AMPK signaling pathway and inhibited ER stress signaling pathways (Fig. 3). We observed the mRNA expression of these pathways *in vitro*. Compared with the control group, the *AMPK* expression was significantly decreased. However, the ER stress-related genes, including *GRP78*, *CHOP*, *ATF6* and *IRE1*, were significantly increased in PA-induced hepatocyte steatosis. DHA application effectively ameliorated this trend (Figs. 6A and B, $P \leq 0.05$). Compared with the PA group, the 100 μM DHA supplementation reduced lipid accumulation. Nevertheless, these DHA effects could be confined using CC (Fig. 6C, $P \leq 0.05$). After DHA treatment, similar constraints of CC were also observed on the inflammation, hepatic lipogenesis and ER stress-related gene levels, including *NF κ B*, *TNF α* , *IL-1 β* , *IL-6*, *SCD1*, *C/EBP α* and *CHOP* (Fig. 6D, $P \leq 0.05$).

4. Discussion

The liver is the primary organ that controls lipid homeostasis through complex signaling, biochemical and cellular pathways [34]. However, excess lipid accumulation in hepatic cells affects its functions and evokes diseases, including hepatic steatosis, inflammation, cell death and fibrosis [35, 36]. However, it is important to explore the underlying cellular and molecular mechanisms influencing hepatic lipid metabolism.

DHA is an n-3HUFA, which represents the most abundant fish oil component and exhibits various biological activities, such as anti-inflammatory, antioxidant and anti-obesity effects [27, 37, 38]. Previous studies mainly focused on the impact of DHA on mammalian cells [15, 16]. To date, the detailed cellular and molecular mechanisms by which DHA affects lipid metabolism in teleosts remain obscure. Therefore, this study identified a positive DHA effect against hepatic steatosis in the grass carp by improving antioxidant ability, alleviating inflammation and suppressing hepatic lipid synthesis via activating and inhibiting the AMPK and ER stress signaling pathways (Fig. 7).

Serum ALT and AST are the major markers of liver function [39]. There are tissue injuries that cause many transaminases to enter the bloodstream, and they remain relatively stable and low [40, 41]. The serum levels of ALT were reduced significantly in the 1% DHA group in this study. In addition, lower serum LDL-c and HDL-c contents were also observed in the 1% DHA group. In a previous study, serum LDL-c and HDL-c were reduced in C57BL/6 J mice, indicating that liver injury was lessened [42].

Under normal circumstances, the liver processes a mass of FA. Less than 5% of FA is stored in a stable state as TG, and the relatively small amounts of TG stored in the liver are in the form of cytoplasmic lipid droplets (LDs) [34]. The hepatocytes accumulate excess TG contributing to steatosis and balloon cell degeneration, and it is considered a major evoking factor of fatty liver diseases [43, 44]. C/EBP α is a transcription factor that plays a critical regulatory role in lipogenesis. It is an effective therapeutic target for hepatocyte damage [45]. In addition, C/EBP β promotes the transcription of SREBP-1c in the liver and adipocytes [46]. SREBP-1c is the primary transcription factor related to hepatic lipogenesis. It regulates the downstream transcription factors, including the genes encoding rate-limiting enzymes (e.g., FAS and SCD1) [6, 47]. In obese mice induced by diet, it exerted anti-hepatic steatosis effects when the activity of SREBP-1c was inhibited [7]. Similarly, liver-specific SREBP-1c knockout mice indicated that the expression levels of lipogenic-related enzymes, including ACC1, FASN and SCD1, were significantly reduced, and the hepatic lipid content and TG plasma levels were lower than those of wild-type [48]. This study illustrated that the *SREBP1c* expression controlled several downstream-signaling components, including *FAS* and *SD1*, consistent with other studies [6, 7, 47, 48]. Besides, the above components were significantly up-regulated in PA-induction, which subsequently increased the TG content. However, DHA treatment could converse these aberrant trends. Furthermore, *in vivo* studies confirmed that DHA lowered the hepatosomatic index, lipid vacuoles during the H&E staining of liver samples, TG content and the expression of transcription factors related to hepatic lipogenesis in the liver. These results suggested that DHA could suppress hepatic lipogenesis and prevent PA-induced lipotoxicity in the liver.

Studies reported that FAS could induce hepatocyte inflammation, which is highly associated with lipid accumulation in the liver. The fatty liver cell model was established using PA, OA, or a mixture of fatty acids (OA: PA) [6, 29, 49]. Previously, DHA could alleviate OA-induced hepatocyte inflammation in grass carp, thus inhibiting the expression levels of transcription factors, such as *IL-8*, *TNF α* and *NF κ B* [27]. PA is more toxic than other saturated or unsaturated fatty acids [50]. Our study determined whether DHA exerted anti-inflammatory effects in grass carp hepatocytes induced by PA. In our study, PA-induced hepatocyte injury caused hepatocyte lipoapoptosis by increasing the TG cell content and up-regulating

the expression of related genes, such as *TNFA*, *IL-6*, *NFkB* and *IL-1β*, during hepatic inflammation. However, DHA supplementation reversed this trend. We also observed that DHA reduced the *TNFA* and *NFkB* serum contents and the expression of transcription factors related to liver inflammation *in vivo*. These results suggest that DHA exerts anti-inflammatory effects. Notably, specialized pro-resolving lipid mediators derived from DHA or EPA, such as resolvins, protectins and maresins, exert potent anti-inflammatory effects in adipocytes [51, 52]. Thus, this research area needs further exploration.

Generally, hepatic lipid accumulation is connected with ER stress, resulting in the progression of liver injury [53, 54]. When the lipid levels in the liver vary significantly, the ER initiates multiple defense systems, termed as unfolded protein response (UPR), to maintain intracellular homeostasis [55]. ER stress can induce the synthesis of hepatic lipids via several mechanisms. It activates hepatic SREBP-1c, a transcription factor binding its membrane, which stimulates numerous hepatic lipogenesis pathways by activating enzyme encoding genes [56–58]. Similarly, in the intestinal epithelial cells of yellow catfish, the FA activated ER stress and induced TG accumulation. In contrast, pretreatment with 4-PBA, an inhibitor of ER stress, alleviated FA-induced accumulation of TG by reducing the expression levels of *SREBP1c* and *FAS*, which suggests that ER stress mediates FA-induced lipogenesis [33]. This study detected the *in vivo* and *in vitro* expression of ER stress-related genes, including *CHOP*, *GRP78*, *IRE1*, *ATF6*, and *PERK*. DHA inhibited hepatic lipogenesis by reducing the mRNA expression of *SREBP1c* and controlling several downstream-signaling components, including *FAS* and *SD1*. It also inhibited ER stress by decreasing the mRNA expression of *GRP78*, *CHOP*, *ATF6* and *IRE1*. Based on these results, DHA relieves liver lipid accumulation by inhibiting ER stress and preventing liver injury.

This study used the transcriptomics approach to analyze the effects of DHA on the liver of grass carp and explore the mechanisms by which DHA protected against hepatic lipid synthesis and inflammation. We observed that lipid uptake, biological metabolism and AMPK signaling pathways were up-regulated in the 1% DHA group compared with the control group. AMPK is a heterotrimer containing three subunits, a catalytic α-subunit and two regulatory subunits (β and γ). AMPK plays an essential role in controlling lipid metabolism, ameliorating hepatic inflammation and improving lipid peroxidation [6, 48, 59]. Our study hypothesized that AMPK could mediate the mechanisms by which DHA inhibited liver lipid synthesis and alleviated liver inflammation. We confirmed that DHA treatment increased the mRNA expression of *AMPK* *in vivo* and *in vitro*. To verify the potential mechanisms of AMPK in lipid accumulation in the liver, hepatocytes were pre-treated with CC, an AMPK antagonist. We also used CC to evaluate the effects of DHA against hepatic lipid synthesis and inflammation. However, CC eliminated the effect of DHA in inhibiting ER stress signaling by up-regulating *CHOP* mRNA expression, which is a crucial marker for ER stress. Consistent with a previous study on HFD-induced obesity in mice, the present study showed that AMPK inhibited lipid-induced ER stress and TG accumulation in liver hepatocytes [60, 61]. Taken together, these results demonstrate that DHA acts as an AMPK agonist to protect the liver from damage by inhibiting the ER stress signaling pathway. These processes subsequently relieve hepatic lipid synthesis and inflammation.

5. Conclusions

This study identified a novel mechanism for regulating hepatic lipid metabolism in grass carp mediated via DHA treatment. It clarifies that DHA exerts a protective effect on hepatic steatosis by inhibiting ER stress, improving antioxidant ability, relieving hepatic inflammation and inhibiting hepatic lipogenesis in an AMPK-dependent manner. Moreover, our study has emphasized the importance of AMPK and ER stress signaling pathways in regulating hepatic lipid metabolism in grass carp.

Abbreviations

A/G, the ratio of albumin and globulin; ALT, alanine aminotransferase; *AMPK*, AMP activated protein kinase; *AST*, aspartate aminotransferase; *ATF6*, activating transcription factor 6; BL, body length; *C/EBP α* , CCAAT-enhancer-binding protein α ; CAT, catalase; CC, Compound C; CF, condition factor; *CHOP*, CCAAT/enhancer-binding protein homologous protein; DHA, Docosahexaenoic acid; ER stress, Endoplasmic Reticulum stress; FAS, fatty acid synthase; FBW, final body weight; GLB, globulin; GLU, glucose; Gpr120, G Protein Coupled Receptor 120; *GRP78*, glucose-regulated protein 78; H&E staining, hematoxylin-eosin staining; HDL-c, high-density lipoprotein cholesterol; HFD, high-fat diet; HSI, hepatosomatic index; *IL-1 β* , interleukin-1 β ; *IL-6*, interleukin-6; IR, insulin resistance; *IRE1*, inositol-requiring enzyme; JNK, Jun N-terminal Kinase; KI, Kidney index; LA, linoleic acid; LDL-c, low-density lipoprotein cholesterol; LNA, linolenic acid; MDA, malondialdehyde; MUFA, monounsaturated fatty acid; n-3 HUFA, n-3 highly unsaturated fatty acids; n-3 PUFA, n-3 polyunsaturated fatty acids; n-3PUFA, n-3 polyunsaturated fatty acids; n-3PUFA/n-6PUFA, the ratio of n-3 polyunsaturated fatty acids and n-6 polyunsaturated fatty acids; n-6 PUFA, n-6 polyunsaturated fatty acids; *NF κ B*, nuclear factor kappa-B; OA, oleic acid; PA, palmitic acid; *PERK*, protein kinase RNA-like ER kinase; PPAR γ , peroxisome proliferator-activated nuclear receptor- γ ; RIL, relative intestine length; *SCD1*, stearoyl-CoA desaturase 1; SFA, saturated fatty acids; SI, Spleen index; SOD, superoxide dismutase; SR, Survival rate; *SREBP1C*, sterol regulatory element-binding protein1c; TG, Triglyceride; TGF β , transforming growth factor- β ; *TNF α* , tumor necrosis factor- α ; TP, total protein; ALB, albumin; UPR, unfolded protein response; VSI, Viscerosomatic index.

Declarations

Ethics approval and consent to participate

The experimental protocol of this study was approved by the Northwest A&F University Institutional Animal Care and Use Committee.

Consent for publication

All authors read and approved the final manuscript, and consent for publication.

Availability of data and material

The data are shown in the main manuscript.

Competing interests

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Authors' contributions

The authors' responsibilities were as follows-Ji Hong, Sun Jian and Huang Xiaocheng: contributed to the conception and design of the study; Huang Xiaocheng, Bian Chenchen and Ji Shanghong: conducted the experiment and sample analysis; Huang Xiaocheng, Ji Hong and Sun Jian: analyzed the data; Huang Xiaocheng: drafted the manuscript; Ji Hong, Sun Jian, Bian Chenchen and Ji Shanghong: revised the manuscript; All authors: contributed to interpreting the results, and read and approved the final manuscript.

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Tables

Table1. Formulation and proximate composition of experimental diets.

Components	Group		
	Control	0.5%DHA	1%DHA
Ingredients (g/kg)			
Casein	320	320	320
Gelatin	80	80	80
Dextrin	280	280	280
Cellulose	189	189	189
Soybean oil	11	11	11
Linseed oil	19	19	19
Lard oil	30	20	10
DHA	0	10	20
Carboxymethylcellulose	20	20	20
Mineral mix ¹	40	40	40
Vitamin mix ²	10	10	10
BHT	1	1	1
Total	1000	1000	1000
Chemical composition (percentage of dry matter basis)			
Crude fat (%)	5.42	5.66	5.60
Crude protein (%; N% × 6.25)	36.48	36.12	36.0
Moisture (%)	9.80	9.98	9.56
Ash (%)	5.83	5.86	5.89

¹ The mineral mix contained (g/100 g of the total mineral) the following ingredients: KAl (SO₄)₂, 0.159; CaCO₃, 18.101, Ca(H₂PO₄)₂, 44.601; CoCl, 0.070; MgSO₄, 5.216; MnSO₄·H₂O, 0.070; KCl, 16.553; KI, 0.014; ZnCO₃, 0.192; NaH₂PO₄, 13.605; Na₂SeO₃, 0.006; CuSO₄·5H₂O, 0.075; Ferric citrate, 1.338.

² The vitamin mix contained (mg/1000 g of diet) the following ingredients: vitamin C, 200; thiamine, 10; riboflavin, 20; vitamin A, 3000IU; vitamin E, 50IU; vitamin D3, 1500IU; menadione, 10; pyridoxine HCl, 10; cyanocobalamin, 0.02; biotin, 1.0; calcium pantothenate, 40; folic acid, 5; niacin, 20; inositol, 400; choline chloride, 2000; cellulose was used as a carrier.

Table 2. Primers used in qPCR analysis.

gene	Forward (5'-3')	Reverse (5'-3')
<i>SREBP1c</i>	TCACTCCAAATGGTGGTCGTCAGT	AAAGTGTTTCAGACAGGCTTTGGGC
<i>FAS</i>	CCTCAGCTTACAGCAGAATC	CTCTTCAGCAAGGGAGTTTAG
<i>C/EBPα</i>	ACCCACATACCACCACTCTCAACA	TTTCCCTCGATCGCCCATCTTCAT
<i>SCD1</i>	GCCTTCCAGAATGACATCTAC	GCCGATGTGAGCAAAGAA
<i>NFκB</i>	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG
<i>TNFα</i>	CGCTGCTGTCTGCTTCAC	CCTGGTCCTGGTTCACTC
<i>IL-6</i>	AGCCAGCTCCAGGTGAGTGAAG	GACGGCTCTGCATGTGTCGATC
<i>IL-1β</i>	GCCAAGTAGCCGAATCACAGA	AAGCCCAAGATATGCAGGAGTC
<i>IL-8</i>	ATGAGTCTTAGAGGTCTGGGT	ACAGTGAGGGCTAGGAGGG
<i>ATF6</i>	AGCTGGAGGATCTTGGGGAT	TGATCGAGGGCTACTCCACA
<i>CHOP</i>	GCGTGGTATGAAGACCTACAG	CGCTGACCATCGTTTCCA
<i>GRP78</i>	TTCAAGCCGGAGTTCTGTCC	GTTGTCAGAAGCGGTGGAGA
<i>IRE1</i>	CCTGCATGGTAGACATCTTCTC	TTCTCTGGCTCCATGCTTAAC
<i>PERK</i>	AAGGTCATGCGAGAGGTAAAG	CATGAGAAGTCCACTGGAGAAG
<i>SOD</i>	GGAGACCTTGTAATGTGATAG	CATGGTCCTCCCAATGATG
<i>CAT</i>	GACTAAGGTCTGGTCCCATAA	TGTTCAACCTCAGCGAAATAG
<i>AMPKα1</i>	TAGTGGCCGAGACTCAACCG	TCTGGCTACGGATTCCCAGG
<i>AMPKα2</i>	AGTTCTACCTGGCCTCCAGT	GAGAGCATCCAGAGGGCATC
<i>β-Actin</i>	TCCACCTTCCAGCAGATGTGGATT	AGTTTGAGTCGGCGTGAAGTGGTA

Abbreviations: *AMPKα1*, AMP activated protein kinase 1; *AMPKα2*, AMP activated protein kinase 2; *ATF6*, activating transcription factor 6; *C/EBPα*, CAAT enhancer binding protein α; *CHOP*, CCAAT/enhancer-binding protein homologous protein; *FAS*, fatty acid synthase; *GRP78*, glucose-regulated protein 78; *IL-1β*, interleukin-1β; *IL-6*, interleukin-6; *IL-8*, interleukin-8; *IRE1*, inositol—requiring enzyme; *NFκB*, nuclear factor kappa-B; *PERK*, protein kinase RNA—like ER kinase; *SCD1*, stearyl-CoA desaturase 1; *SOD*, superoxide dismutase; *CAT*, catalase; *SREBP1c*, sterol regulatory element-binding protein-1c; *TNFα*, tumor necrosis factor-α.

Table 3. Fatty acid composition of the experimental diets (% total fatty acids).

Components	Group		
	Control	0.5%DHA	1%DHA
14:0	1.0	1.6	1.3
16:0	18.2	15.3	12.0
18:0	12.0	9.9	7.2
SFA	31.1	26.7	20.5
16:1n-7	1.3	1.2	0.8
18:1n-9c	30.3	27.1	21.6
MUFA	31.6	28.3	22.3
18:2n-6	19.3	18.7	18.6
18:3n-6	0.5	0.5	0.8
20:4n-6	0.2	0.7	0.6
22:4n-6	0.2	0.7	1.5
n-6PUFA	20.1	20.5	21.6
18:3n-3	16.4	17.1	19.1
20:5n-3	0.1	1.2	2.6
22:6n-3	0.1	7.6	16.9
n-3HUFA	0.2	8.7	19.4
n-3PUFA	16.6	25.8	38.5
PUFA	36.7	46.3	60.1
n-3/n-6PUFA	0.8	1.3	1.8

Abbreviations: MUFA, monounsaturated fatty acid; n-3 PUFA, n-3 polyunsaturated fatty acids; n-3PUFA, n-3 polyunsaturated fatty acids; n-3PUFA/n-6PUFA, the ratio of n-3 polyunsaturated fatty acids and n-6 polyunsaturated fatty acids; n-6 PUFA, n-6 polyunsaturated fatty acids; SFA, saturated fatty acids.

Table 4. Effect of dietary DHA on growth performance and biological indices of grass carp¹.

Parameter	Group		
	Control	0.5%DHA	1%DHA
IBW (g)	29.8 ± 0.22	29.8 ± 0.01	29.8 ± 0.02
FBW (g)	39.4 ± 1.55 ^b	39.5 ± 1.16 ^b	43.0 ± 1.37 ^a
BL (cm)	12.8 ± 0.08 ^b	13.0 ± 0.05 ^b	13.3 ± 0.17 ^a
RIL ² (%)	178 ± 2.80 ^a	172 ± 1.50 ^{ab}	169 ± 0.13 ^b
CF ³ (g/cm ³)	1.89 ± 0.05	1.82 ± 0.01	1.83 ± 0.02
SR ⁴ (%)	94.5 ± 4.81	97.2 ± 4.81	100 ± 0.00
VSI ⁵ (%)	14.7 ± 0.54 ^a	14.1 ± 0.27 ^{ab}	13.7 ± 0.19 ^b
KI ⁶ (%)	0.32 ± 0.01	0.31 ± 0.01	0.33 ± 0.01
SI ⁷ (%)	0.13 ± 0.05	0.10 ± 0.01	0.09 ± 0.00
HSI ⁸ (%)	2.95 ± 0.03 ^a	3.09 ± 0.13 ^a	2.62 ± 0.11 ^b

Abbreviations: BL, body length; CF, condition factor; FBW, final body weight;

HSI, hepatopancreas index; IBW, initial body weight; KI, kidney index; RIL, relative intestine length; SI, spleen index; SR, survival rate; VSI, viscerosomatic index.

¹Values are expressed as mean ± standard deviation (SD), n =3 (randomly picked 10 fish from each tank, and the average of each tank is calculated, three replicates per treatment). Labeled means in a row without a common letter are significantly different, $P \leq 0.05$ [1-factor analysis of variance (ANOVA) followed by Duncan's test].

²Relative intestine length (RIL, %) = intestine length (cm) × 100/ body length (cm)

³Condition factor (CF, g/cm³) = body weight (g) × 100/ body length (cm³)

⁴Survival rate (SR, %) = final number of fish × 100/initial number of fish

⁵Viscera index (VSI, %) = viscera weight (g) × 100/body weight (g)

⁶Kidney index (KI, %) = kidney weight (g) × 100/body weight (g)

⁷Spleen index (SI, %) = spleen weight (g) × 100/body weight (g)

⁸Hepatopancreas index (HSI, %) = hepatopancreas weight (g) × 100/body weight (g)

Table 5. Effect of dietary DHA on serum biochemical parameters of grass carp¹.

Index	Group		
	Control	0.5%DHA	1%DHA
TP (g L ⁻¹)	24.32±2.86	23.5±3.59	20.6±2.44
ALB (g L ⁻¹)	11.14±1.32 ^a	10.78±1.70 ^{ab}	9.23±0.89 ^b
GLO (g L ⁻¹)	13.18±1.59	12.72±1.92	11.36±1.57
A/G	0.85±0.04	0.85±0.03	0.82±0.05
GLU (mmol L ⁻¹)	5.90±1.17	5.51±1.46	5.04±0.98
HDL-c (mmol L ⁻¹)	0.73±0.10 ^a	0.82±0.10 ^a	0.61±0.04 ^b
LDL-c (mmol L ⁻¹)	1.43±0.26 ^b	1.89±0.42 ^a	1.01±0.14 ^c

¹Values are expressed as mean ± standard deviation (SD), n = 6 fish (2 fish/tank). Labeled means in a row without a common letter are significantly different, $P \leq 0.05$ [1-factor analysis of variance (ANOVA) followed by Duncan's test].

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase;

A/G, albumin/ globulin; ALB, albumin; GLO, globulin;

HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; TP, total protein.

Table 6. Effect of dietary DHA on the fatty acid composition of the liver in grass carp¹ (% total fatty acid).

Components	Group		
	Control	0.5%DHA	1%DHA
14:0	1.98 ± 0.23 ^a	1.67 ± 0.18 ^b	1.77 ± 0.05 ^{ab}
16:0	24.4 ± 2.07 ^a	21.6 ± 0.64 ^b	17.2 ± 1.64 ^c
18:0	6.28 ± 0.78 ^a	6.62 ± 1.16 ^a	3.95 ± 0.44 ^b
SFA	31.6 ± 0.89 ^a	29.9 ± 1.16 ^a	22.9 ± 2.04 ^b
16:1n-7	10.0 ± 1.33	9.34 ± 0.90	11.2 ± 0.85
18:1n-9c	47.5 ± 1.62	48.7 ± 3.58	48.5 ± 2.06
MUFA	57.5 ± 2.02	58.0 ± 4.20	59.7 ± 2.70
18:2n-6	3.08 ± 0.78	3.45 ± 1.45	4.13 ± 1.08
18:3n-6	0.15 ± 0.04	0.15 ± 0.06	0.17 ± 0.03
20:4n-6	1.34 ± 0.46	0.95 ± 0.37	1.25 ± 0.08
22:4n-6	0.43 ± 0.07 ^b	0.51 ± 0.10 ^b	0.90 ± 0.22 ^a
n-6PUFA	5.62 ± 0.93	5.67 ± 1.74	7.10 ± 1.40
18:3n-3	3.07 ± 0.39	3.25 ± 1.34	4.44 ± 0.66
20:5n-3	0.50 ± 0.18 ^a	0.26 ± 0.15 ^b	0.24 ± 0.04 ^b
22:6n-3	1.60 ± 0.42 ^c	2.94 ± 1.02 ^b	5.26 ± 0.74 ^a
n-3HUFA	2.10 ± 0.58 ^b	3.20 ± 1.34 ^b	5.50 ± 0.74 ^a
n-3PUFA	5.17 ± 0.52 ^b	6.45 ± 2.34 ^b	9.93 ± 1.16 ^a
PUFA	10.8 ± 1.33 ^b	12.1 ± 4.03 ^b	17.0 ± 2.17 ^a
n-3/n-6PUFA	0.92 ± 0.56 ^b	1.13 ± 0.14 ^b	1.43 ± 0.30 ^a

¹Values are expressed as mean ± standard deviation (SD), n = 6 fish (2 fish/tank). Labeled means in a row without a common letter are significantly different, $P \leq 0.05$ [1-factor analysis of variance (ANOVA) followed by Duncan's test].

Abbreviations: MUFA, monounsaturated fatty acid; n-3 PUFA, n-3 polyunsaturated fatty acids; n-3PUFA, n-3 polyunsaturated fatty acids; n-3PUFA/n-6PUFA, the ratio of n-3 polyunsaturated fatty acids and n-6 polyunsaturated fatty acids; n-6 PUFA, n-6 polyunsaturated fatty acids; SFA, saturated fatty acids.

Figures

Figure 1

DHA attenuates lipid accumulation and improves liver antioxidant capacity (Experiment 1). (A) The representative hematoxylin & eosin (H&E) staining of liver sections; (B) hepatosomatic index; (C) TG liver levels; (D-E) the liver contents of MDA, SOD and CAT; (F) the mRNA expression levels of SOD and CAT. Values are expressed as mean $\pm$ standard deviation (SD), n = 6 fish (2 fish/tank). Labeled means without a common letter are significantly different, $P \leq 0.05$ [1-factor analysis of variance (ANOVA) followed by Duncan's test]. CAT, catalase; MDA, maleic dialdehyde; SOD, superoxide dismutase; TG, triglyceride.

Figure 2

DHA inhibits the expression of hepatic lipid synthesis and inflammation-related genes (Experiment 1). (A-B) Serum levels of NFκB and TNFα; (C-D) the mRNA expression of TNFα, NFκB, IL-1β, IL-6 and IL-8 in the liver; (E-F) the mRNA expression of SREBP1c, C/EBPα, FAS and SCD1 in the liver. Values are expressed as mean ± standard deviation (SD), n = 6 fish (2 fish/tank). Labeled means without a common letter are significantly different, $P \leq 0.05$ [1-factor analysis of variance (ANOVA) followed by Duncan's test].

Figure 3

DHA activates AMPK and inhibits ER stress signaling pathways to influence hepatic lipid metabolism (Experiment 1). (A) The up-regulated EuKaryotic Orthologous Group (KOG) biological pathways in the 1% DHA group when compared with the control group (n = 3); (B) the up-regulated Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathways in 1% DHA group relative to the control group (n = 3); (C) the AMPK mRNA expression (n=6 fish, 2 fish/tank); (D-E) the mRNA expression of CHOP, GRP78, IRE1, ATF6 and PERK (n=6 fish, 2 fish/tank). Values are expressed as mean $\pm$ standard deviation (SD). Labeled means without a common letter are significantly different, $P \leq 0.05$ [1-factor analysis of variance (ANOVA) followed by Duncan's test]. *Different from control, $P \leq 0.05$ (Student t test).

Figure 4

DHA affects cell viability and PA-induced lipid accumulation in hepatocytes (Experiment 2). (A) A representative of cell Nile red staining; (B) cell TG; (C) the mRNA expression of FAS, SCD1 and SREBP1c; (D) the mRNA expression of IL-6, TNFα, NFκB and IL-1β; (E) cell viability. Values are expressed as mean ± standard deviation (SD), $n \geq 3$ (cell TG, $n=4$; the mRNA expression, $n=3$; cell viability, $n=6$). Labeled means without a common are significantly different, $P \leq 0.05$ [1-factor analysis of variance (ANOVA) followed by Duncan's test]. *Different from control, $P \leq 0.05$ (Student t test).

Figure 5

DHA ameliorates PA-induced inflammation and lipid synthesis in hepatocytes (Experiment 2). (A) A representative of cell Nile red staining; (B) cell TG; (C) the mRNA expression of FAS, SREBP1c, SCD1 and C/EBPα; (D) the mRNA expression of TNFα, IL-6, NFκB and IL-1β; (E) western blot analysis on FAS expression. Values are expressed as mean ± standard deviation (SD), n≥3 (cell TG, n=4; the mRNA expression, n=3). Labeled means without a common letter are significantly different, P<0.05 [1-factor analysis of variance (ANOVA) followed by Duncan's test].

Figure 6

DHA ameliorates hepatic lipid synthesis and inflammation via the AMPK signaling pathway in hepatocytes (Experiment 2). (A) The AMPKα1 and AMPKα2 mRNA expression; (B) the mRNA expression of different ER stress genes, including GRP78, CHOP, ATF6 and IRE1; (C) cell TG; (D) the mRNA expression of genes involved in inflammation, lipogenesis and ER stress. Values are expressed as mean $\pm$ standard deviation (SD), $n \geq 3$ (cell TG, $n=4$; the mRNA expression, $n=3$). Labeled means without a common letter are significantly different, $P \leq 0.05$ [1-factor analysis of variance (ANOVA) followed by Duncan's test].

Figure 7

DHA ameliorates palmitic acid-induced lipid accumulation in grass carp hepatocytes. Therefore, the underlying mechanism could be associated with inhibiting ER stress, improving antioxidant ability, relieving hepatic inflammation and inhibiting hepatic lipogenesis in an AMPK-dependent manner.