Article

A Combination of Flaxseed Oil and Astaxanthin Improves Hepatic Lipid Accumulation and Reduces Oxidative Stress in High Fat-Diet Fed Rats

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Abstract: Hepatic lipid accumulation and oxidative stress are crucial pathophysiological mechanisms for non-alcoholic fatty liver disease (NAFLD). Thus, we examined the effect of a combination of flaxseed oil (FO) and astaxanthin (ASX) on hepatic lipid accumulation and oxidative stress in rats fed a high-fat diet. ASX was dissolved in flaxseed oil (1 g/kg; FO + ASX). Animals were fed diets containing 20% fat, where the source was lard, or 75% lard and 25% FO + ASX, or 50% lard and 50% FO + ASX, or FO + ASX, for 10 weeks. Substitution of lard with FO + ASX reduced steatosis and reduced hepatic triacylglycerol and cholesterol. The combination of FO and ASX significantly decreased hepatic sterol regulatory element-binding transcription factor 1 and 3-hydroxy-3-methylglutaryl-CoA reductase but increased peroxisome proliferator activated receptor expression. FO + ASX significantly suppressed fatty acid synthase and acetyl CoA carboxylase but induced carnitine palmitoyl transferase-1 and acyl CoA oxidase expression. FO + ASX also significantly elevated hepatic SOD, CAT and GPx activity and GSH, and markedly reduced hepatic lipid peroxidation. Thus, FO and ASX may reduce NAFLD by reversing hepatic steatosis and reducing lipid accumulation and oxidative stress.

Keywords: flaxseed oil; astaxanthin; high fat diet; lipid accumulation; oxidant stress

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), which includes benign hepatic steatosis and cirrhosis, has been shown to be associated with obesity, hyperlipidemia and type II diabetes [1,2]. As the prevalence of these diseases is increasing worldwide, NAFLD has become a serious health concern [3]. NAFLD and associated increased prevalence of coronary artery disease (CAD) [4] contribute to more morbidity and mortality and create greater health-care costs. The pathogenesis of NAFLD is thought to occur via a "double-hit": hepatocellular lipid accumulation and subsequent oxidative stress [5].

N-3 polyunsaturated fatty acids (*n*-3 PUFAs) have been of interest because of their purported benefits for many chronic diseases including cardiovascular disease, cancer, and type II diabetes [6]. The plant-derived *n*-3 PUFA, α-linolenic acid (ALA), is nutritionally essential and cannot be synthesized de novo in vertebrate tissue [7]. ALA can compete with linoleic acid to reduce arachidonic acid or act as a precursor for longer chain *n*-3 PUFAs (such as EPA and DHA), or it can directly interact with nuclear receptors and ion channels to produce positive effects [8]. Because it is high in ALA, flaxseed oil (FO) may be a promising functional food ingredient. Recently, ALA has been shown to lower hepatic lipids and reduce NAFLD [9]. However, ALA is particularly susceptible to oxidation, so addition of FO may increase hepatic lipid peroxidation [10], which may adversely affect hepatoprotection.

As a lipophilic xanthophyll carotenoid, astaxanthin (ASX) is produced de novo by microorganisms and plants, but it is also found in diverse microalgae, fungi and crustaceans. ASX has a hydroxyl (OH) and keto (CdO) moiety on each ionone ring and its molecular structure lends it powerful antioxidant activity [11]. Recently, ASX has been studied for its anti-inflammatory, anti-tumor, anti-diabetic, cardiovascular and neuroprotective activities as well for its ability to modulate immune function [11–15]. ASX has been shown to protect against hepatic endoplasmic reticular stress, inflammation and lipid deposition in high fat, high-fructose fed mice [16,17], as well as protect against dioxin-induced hepatotoxicity [18]. However, the effectiveness of administration of antioxidants alone is suboptimal to inhibit the progression of NAFLD [19]. We previously reported that FO and ASX reduced plasma oxidative stress, lipids, and inflammation, and may contribute to cardiovascular protection [20]. Thus, we studied whether this combination can reduce liver damage in rats fed a high-fat diet.

2. Methods

2.1. Chemical Sources

Flaxseed oil was obtained from Caoyuankangshen Food Co., Ltd. (Inner Mongolia, China). Commercial deodorized lard was purchased from a local supermarket. Astaxanthin was obtained from Jingzhou Natural Astaxanthin Inc. (Jingzhou, China) and was dissolved in flaxseed oil to a final concentration of 1 g/kg (FO + ASX) when used.

2.2. Animals and Diets

Forty male Sprague–Dawley rats (initially 150–170 g) were purchased from Sino-British Sippr/BK (Shanghai, China). Animals were housed individually and maintained at a controlled ambient temperature (22 ± 1 °C) under diurnal conditions (light–dark: 08:00–20:00) with access to laboratory chow and tap water *ad libitum*. After 1 week of acclimatization, rats were randomized into a high-fat diet (CON) group and three experimental groups (*n* = 10 animals/group). All animals were fed purified experimental diets as shown in Table 1. Dietary fat was provided by lard (CON group), or 75% lard and 25% FO + ASX (L-FO + ASX group), or 50% lard and 50% FO + ASX (M-FO + ASX group), or 100% FO + ASX (H-FO + ASX group). Weekly purified diets were mixed, formed into a dough with purified water, rolled into pellets, sealed in air-tight plastic bags under nitrogen gas and stored at −80 °C until use. Food in the cages was shaded from light and changed daily. All animals were weighed twice a week and food intake was measured weekly. Animals were cared for in accordance with The Guiding Principles in the Care and Use of Animals. The experiment was approved by the

Oil Crops Research Institute Council on Animal Care Committee, Chinese Academy of Agricultural Sciences ((2013) IACUC Number: 0021).

Table 1. Nutrient content and ingredient composition of the experimental diets.

Diet	g	kcal
Nutrient,%		
Protein	20	17
Carbohydrate	50	43
Fat	20	39
Total		100
Ingredient		
Casein	200	800
DL-methionine	3	12
Maize starch	350	1400
Sucrose	150	600
Cellulose	50	0
Mineral mixture (AIN-93M)	35	0
Vitamin mixture (AIN-93M)	10	40
Choline bitartrate	2	0
Fat	200	1800
Total	1000	4652

2.3. Tissue Preparation

After 10 weeks of treatment, rats were fasted for 16 h and then killed under anesthesia. Then, cardiac blood was collected with sodium heparin and centrifuged at $1500 \times g$ for 10 min at 4 °C to remove plasma which was stored at -80 °C. Livers were rapidly dissected, weighed, and a small piece of the right liver lobe was fixed in 4% paraformaldehyde for light microscopy. Remaining liver tissue was stored at -80 °C until analysis.

2.4. Serum Assays

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantified using a Hitachi 7020 auto-analyzer with commercial kits (Wako Pure Chemicals, Osaka, Japan).

2.5. Liver Histology

Liver was sectioned at 5 μm thickness and stained with hematoxylin and eosin (H&E). Fifteen digital images per animal were selected for estimating volume density (Vv) of liver steatosis by point-counting hepatic fat droplets as previously described [21].

2.6. Liver Lipid Content

Lipids were extracted from 1 g liver with a mixture of chloroform/methanol (2:1, *v/v*) by the method of Folch [22]. Hepatic triglyceride (TG) and total cholesterol (TC) were measured with commercial kits (Zhongsheng Beikong Biotech Company, Beijing, China).

2.7. Western Blot

Liver samples were lysed in ice-cold RIPA buffer supplemented with 1 mM PMSF (Sigma, St. Louis, MO, USA) and then kept at 4 °C for 2 h. Supernatant was collected by centrifuge at $10,000 \times g$ for 15 min at 4 °C. For SDS–PAGE, samples were mixed with SDS sample buffer and incubated at 98 °C for 5 min. Western blot was performed with the following antibodies: β-actin mAb (#3700, Cell Signaling, Danvers, MA, USA), peroxisome proliferator activated receptor α (PPARα) pAb (ab8934, Abcam, Cambridge, UK), sterol regulatory element-binding transcription factor 1 (SREBP1)

mAb (sc-365513, Santa Cruz Technology, Santa Cruz, CA, USA), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) mAb (ab174830, Abcam).

2.8. Quantitative Real-Time PCR

Total RNA of the liver was isolated using Trizol (Invitrogen, Grand Island, NY, USA) as indicated by kit instructions. Using the RT system (Takara Bio, Dalian, China), cDNA was synthesized from total RNA. Real-time PCR was performed with SYBR Premix Ex Taq II (Takara Bio, Dalian, China), using the ABI 7900HT real-time thermocycler (Applied Biosystems, Forster, CA, USA). The dissociation curve of each gene was performed and analyzed using ABI 7900HT software, and the result confirmed product specificity. Each sample was analyzed three times and normalized to β-actin. Results of real-time PCR were analyzed with the $2^{-\Delta\Delta Ct}$ method as previously described [23]. Primer sequences for genes in this study were as follows. fatty acid synthase (FAS): forward 5′-GGACATGGTCACAGACGATGAC-3′, reverse 5′-GTCGAACTTGGACAGATCCTTCA-3′; acetyl CoA carboxylase (ACC): forward 5′-GCCTCTTCCTGACAAACGAG-3′, reverse 5′-TCCATACGCCTGA AACATGA-3′; carnitine palmitoyl transferase-1 (CPT-1): forward 5′-AACTTTGTGCAGGCC ATGATG-3′, reverse 5′-AGCTTGTGAGAAGCACCAGCA-3′; acyl CoA oxidase (ACO): forward 5′-ATCTCTGTGGTTGCTGTGGAGTCA-3′, reverse 5′-TCTGGATG CTTCCTTCTCCAAGGT-3′.

2.9. Assay of Hepatic Antioxidant Activity and Lipid Peroxidation

Livers were weighed and a 10% homogenate was prepared in a 50 mmol/L phosphate buffer (pH 7.0) containing 0.1 mmol/L EDTA. Homogenate was centrifuged at $1000\times g$ for 10 min at 4 °C for subsequent tests. Superoxide dismutase (SOD) activity was measured according to the method of Kono [24]. Glutathione peroxidase (GPx) activity was assayed by the method of Sazuka [25]. Catalase (CAT) activity was estimated using the method of Goth [26]. Glutathione (GSH) was quantified using methods published by Moron [27]. Thiobarbituric acid reactive substances (TBARS) were quantified using the method of Buege [28], and all methods used have been previously described [29].

2.10. Protein Measurement

Protein was quantified using the Lowry method [30] and bovine serum albumin (BSA) as a standard.

2.11. Statistical Analyses

Results were expressed as means ± SEM (standard error of the mean), and analyses were based on one-way ANOVA, followed by the Fisher PLSD *post hoc* test if differences were significant. All statistical analyses were performed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA), and the limit of statistical significance was set at $p < 0.05$.
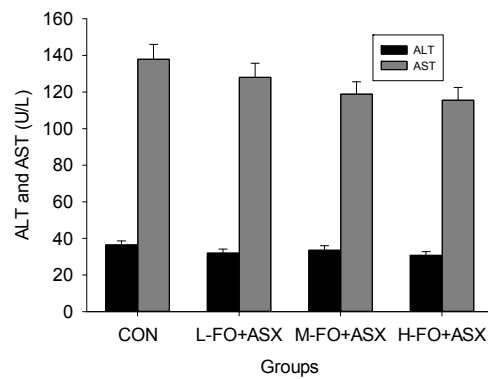
3. Results

3.1. Food Intake and Body Weight Gain

There were no differences in food intake and weight gain among all groups during the study period (data not shown).

### 3.2. Effects of FO and ASX Combination on Hepatic Enzymes in Plasma

Plasma ALT and AST decreased in all FO + ASX groups (Figure 1), but these changes never reached statistical significance ($p > 0.05$).

Figure 1. Effects of flaxseed oil (FO) and astaxanthin (ASX) on plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in rats fed a high-fat diet. CON: high-fat diet group; L-, M- and H-FO + ASX: low, moderate, and high FO and ASX groups. Bars represent means ± SEM (n = 10/group).

3.3. Effects of FO and ASX on Liver Morphology

Photomicrographs of H&E-stained liver sections appear in Figure 2. Rats fed a high-fat diet for 10 weeks developed extensive microvesicular steatosis and scattered macrovesicular steatosis. However, when rats received FO + ASX, circular lipid droplets were reduced and even fewer were present in H-FO + ASX animals. Steatosis was less in all treated animals fed a high-fat diet as compared to controls.
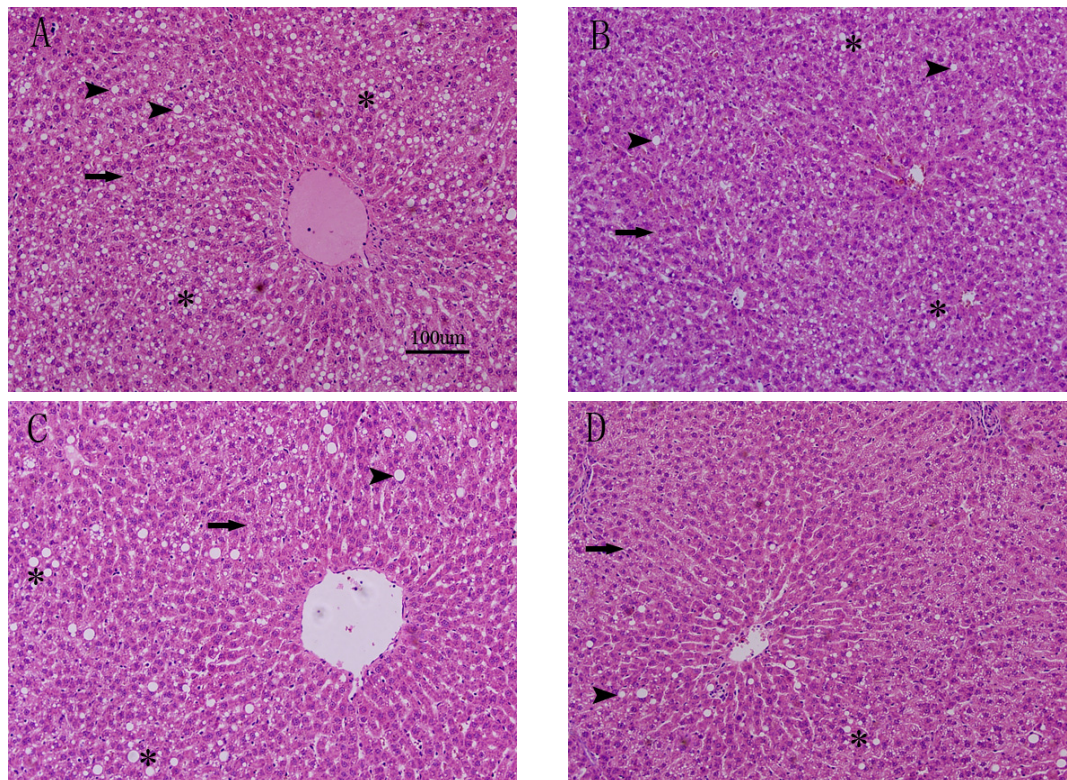

Figure 2. *Cont.*
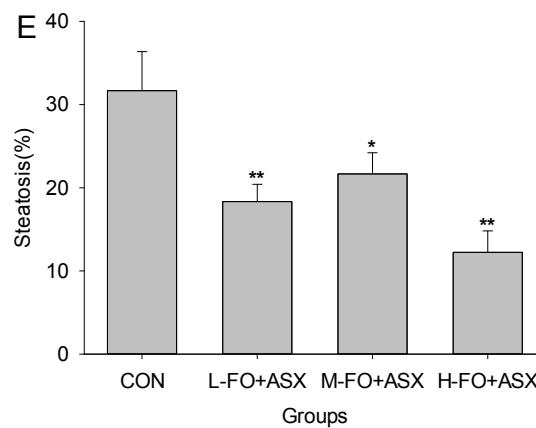
Figure 2. Liver histology after hematoxylin and eosin (H&E) staining of sections from representative rats from each group: (**A**) high-fat diet group; (**B**) low FO and ASX group; (**C**) moderate FO and ASX group and (**D**) high FO and ASX group. Arrow/arrowheads indicate micro- and macrovesicular steatosis, respectively. Asterisks mark scattered foci of macrovesicular steatosis; Volume density quantitation of hepatic steatosis (**E**). Bars represent means $\pm$ SEM (n = 5 animals/group). * $p < 0.05$ and ** $p < 0.01$ compared to CON group.

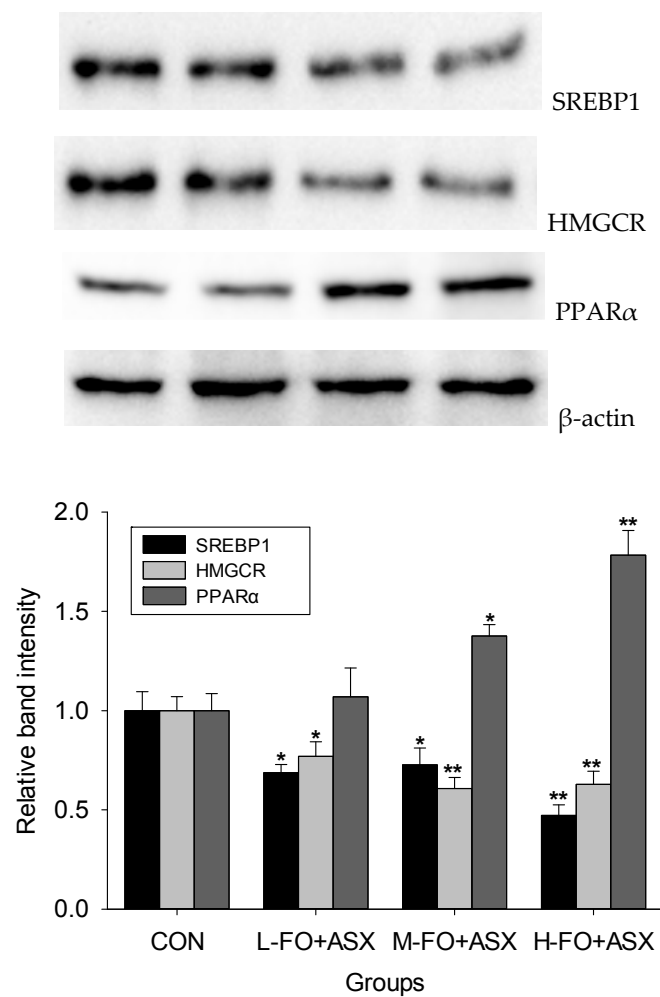## 3.4. Effects of FO and ASX on Liver Lipids

As shown in Figure 3, hepatic TGs were lower in the L-FO + ASX, M-FO + ASX, and H-FO + ASX group than in controls. Similarly, livers from M-FO + ASX and H-FO + ASX animals had less TC compared to control rats.
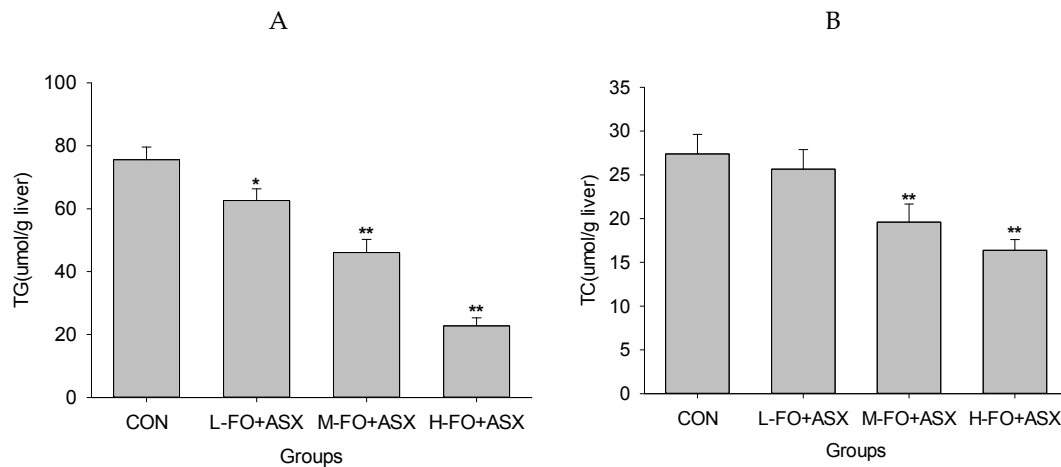
Figure 3. Effects of FO and ASX on hepatic triglyceride (TG) (**A**) and total cholesterol (TC) (**B**) in rats fed a high-fat diet. CON: high-fat diet group; L-, M- and H-FO + ASX: low, moderate, and high FO and ASX groups. Bars represent means $\pm$ SEM (n = 10 animals/group). * $p < 0.05$ and ** $p < 0.01$ compared to CON group.

## 3.5. Effects of FO and ASX on Liver Protein Expression

Compared to controls (Figure 4), all FO + ASX had less SREBP1 and HMGCR protein expression but more PPARα protein expression was observed in M-FO + ASX and H-FO + ASX groups.

Figure 4. Effects of FO and ASX on hepatic lipid synthesis related proteins expression. Representative Immunoblot comparing relative SREBP1, HMGCR and PPARα protein. CON: high-fat diet group; L-, M- and H-FO + ASX: low, moderate, and high FO and ASX groups. Bars represent means ± SEM from 4 independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared to CON group.

### 3.6. Effects of FO and ASX on Liver mRNA Expression

FAS and ACC, two important SREBP-1 target enzymes [31], are essential for fatty acid synthesis. Figure 5 show that FAS mRNA expression in the H-FO + ASX group and ACC in the M-FO + ASX and H-FO + ASX groups were lower than in controls. Conversely, H-FO + ASX significantly increased CPT-1 and ACO mRNA expression; these are PPAR α-induced rate-limiting enzymes of fatty acid oxidation [32,33].
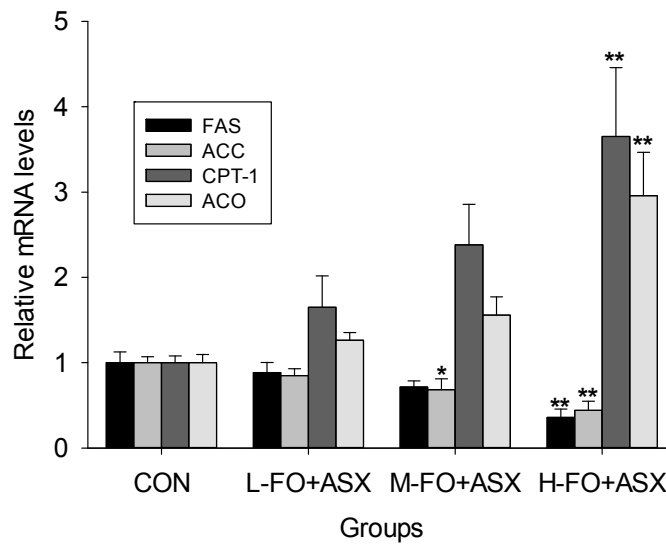
Figure 5. Effects of FO and ASX on hepatic FAS, ACC, CPT-1 and ACO mRNA. CON: high-fat diet group; L-, M- and H-FO + ASX: low, moderate, and high FO and ASX groups. Bars represent means ± SEM from 4 independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared to CON group.

3.7. Effects of FO and ASX on Liver Antioxidant Capacity and Lipid Peroxidation

Figure 6 shows that SOD activity in the H-FO + ASX group was increased compared to controls, as was CAT activity in all FO + ASX groups. GPx activity was greater in the M-FO + ASX and H-FO + ASX groups. After M-FO + ASX and H-FO + ASX treatment, GSH was elevated compared with controls. TBARS were lower in the M-FO + ASX and H-FO + ASX groups compared to controls.

Figure 6. *Cont.*

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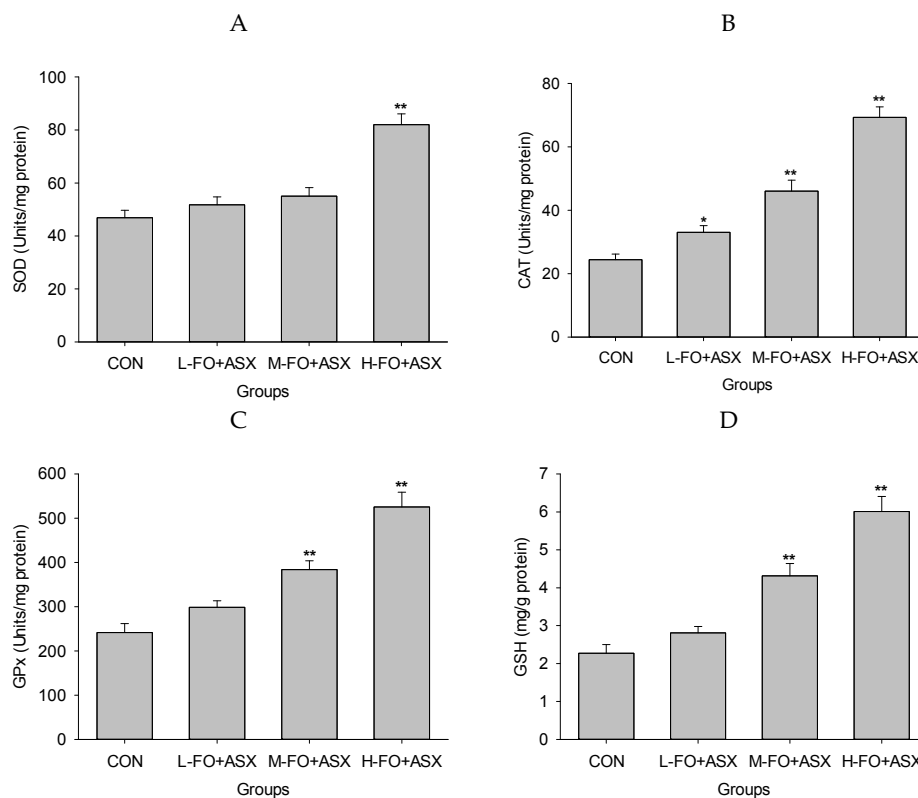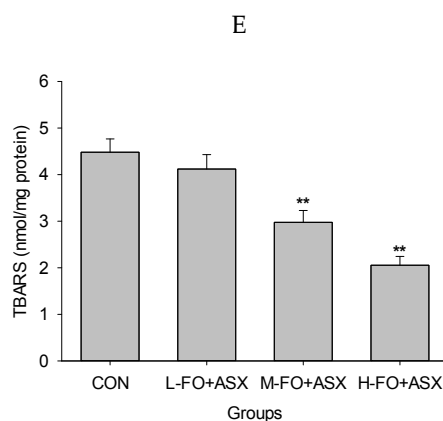
Figure 6. Effects of FO and ASX on SOD (**A**), GPx (**B**) and CAT activity (**C**), GSH (**D**), and TBARS (**E**) in liver of rats fed a high-fat diet. CON: high-fat diet group; L-, M- and H-FO + ASX: low, moderate, and high FO and ASX groups. Bars represent means ± SEM (*n* = 10 animals/group). * *p* < 0.05 and ** *p* < 0.01 compared to CON group.

4. Discussion

Much progress has been made to elucidate the pathogenesis of NAFLD. The most widely accepted mechanism for NAFLD is a "two-hit" theory [5]: the first hit being excessive hepatic fat accumulation which develops when an imbalance in fatty acid uptake and de novo lipogenesis exceeds oxidation and exportation [34]. The second hit is a hepatocellular injury due to oxidative stress, which drives the disease development. A high-fat diet induces significant lipid accumulation and oxidative stress [35], and may cause many chronic metabolic diseases [36] including NAFLD [37]. Liver histological analysis is used to evaluate the presence and severity of NAFLD, and this was used to show that a high-fat diet caused noticeable hepatic steatosis, the earliest stage of NAFLD. FO + ASX treatment diminished the severity of hepatic steatosis, and M- and H-FO + ASX significantly reduced hepatic triacylglycerol and cholesterol. Thus, reducing hepatic lipid accumulation is key (an important mechanism) for the combination of FO and ASX to reversing initial NAFLD. ALA can reduce fatty acid synthesis by suppressing numerous lipogenic enzymes, such as FAS and lipogenic transcriptional factor SREBP1 [38,39]. PPARα is needed to regulate fatty acid metabolism, and ALA is a natural ligand of PPARα, binding to and activating this critical transcriptional regulator to increase gene expression and enzyme activity involved in hepatic fatty acid oxidation [38–42]. ALA has more affinity for carnitine:palmitoyl transferase-1, the rate-limiting enzyme of fatty acid β-oxidation [43], which make ALA a preferential substrate relative to other fatty acids (such as 16:0 and 18:0 saturated fatty acids, oleic acid, linoleic acid) for mitochondrial and peroxisomal β-oxidation pathways [42,44]. Thus, the special spatial arrangement is determined by the number and position of double bonds [43], the greater extent of partitioning of ALA towards β-oxidation rather than deposition of TG. Depletion of hepatic *n*-3 long-chain PUFAs (LCPUFA) should favor TG synthesis over oxidation and may contribute to steatosis of NAFLD [45]. As the precursor fatty acid of *n*-3 LCPUFA, ALA significantly elevates EPA and DHA in hepatic membranes and reduces hepatic TG [46]. In addition, ALA increases CYP7A1 activity, which enhances secretion of hepatic cholesterol into bile and lowers hepatic cholesterol [47]. Similar to other antioxidant nutraceuticals [48], ASX can regulate liver lipid content. Peroxisome proliferator-activated receptor-γ (PPARγ) is thought to be a presteatotic factor in fatty liver [49]. ASX induces effects opposite to that of PPARα (of which it is an agonist) and PPARγ (of which it is an antagonist) [50], which has an overall effect of reducing hepatic lipid accumulation [51,52] by rewiring hepatic lipid metabolic gene expression [50,53]. Moreover, CYP27A1 regulates bile acid synthesis, and ASX can substantially induce CYP27A1 gene expression [50] and maintain hepatic cholesterol homeostasis. FO and ASX decrease hepatic expression of SREBP1 and HMGCR, but increased expression of PPARα. In terms of results, this combination potently depressed FAS and ACC expression and significantly induced CPT-1

and ACO expression. Thus, the combination of FO and ASX may decrease hepatic triacylglycerol and cholesterol by suppressing hepatic lipogenesis and cholesterol synthesis as well as by promoting lipid oxidation.

Data show that high-fat diet diminishes hepatic antioxidant status and elevates oxidative stress and lipid peroxidation [54,55]. After a FO + LA diet, H-FO + ASX treated animals had more SOD, CAT and GPx activity, and more GSH. More antioxidant activity and reduced lipid peroxidation suggested alleviation of hepatic oxidative stress. Although FO may reduce hepatic GSH depletion by lowering cholesterol and triacylglycerol [9], the potent antioxidant properties of ASX should be mainly responsible for the dramatic hepatic oxidative stress improvement effect after FO + LA consumption. Due to polar moieties on both ends of the polyene chain, ASX had more free radical scavenging capability than α-tocopherol or β-carotene [56–58]. Also, structurally damaged mitochondria is a major source of oxidative stress in NAFLD [59], and ASX protects the mitochondrial redox state and its functional integrity [60] to reduce ROS production. In addition, ASX can elevate hepatic antioxidant SOD, CAT and GPx expression and activity by inducing, at least partly, the Nrf2 pathway [52,53] and many non-enzymatic antioxidants such as GSH, vitamins E and C after a high-fat diet or other pathological conditions [16,52,61].

5. Conclusions

FO plus ASX can reduce hepatic steatosis, TG, cholesterol and oxidative stress, suggesting that this may reduce NAFLD induced by a high-fat diet. Thus, FO + ASX may be promising for hepatoprotection but more work is required in additional NAFLD models to confirm this hypothesis.

Author Contributions: Jiqu Xu and Shuang Rong designed the overall research experiments. Hui Gao, Chang Chen, Wei Yang and Qianchun Deng performed the experiments and data analysis. Qingde Huang and Lingyun Xiao collected and analyzed the references. Jiqu Xu and Fenghong Huang wrote and edited the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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