Original Article

Regulation of Adipokines by Polyunsaturated Fatty Acids in a Rat Model of Non-alcoholic Steatohepatitis

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Abstract

Background: Recent evidence has indicated that polyunsaturated fatty acids (PUFA), such as omega-3 PUFA, have protective effects on a range of chronic inflammatory conditions, including obesity, and may play a role in the reversal of steatohepatitis. However, the effects of omega-3 PUFA on adipokine expression and hepatic lipid metabolism have not been well evaluated. Thus, the aim of our study was to investigate the effects of PUFAs on adipokines, as well as lipid and glycometabolism, in a rat model of non-alcoholic steatohepatitis (NASH).

Methods: Male Sprague-Dawley rats were divided into control, model and therapy groups. The control group received a normal diet, while the model and therapy groups received a high-fat diet. On the eighth week of high-fat diet, the therapy group was treated with omega-3 PUFA (1.0 g/d) daily. At the end of 20 weeks, serum biochemistry indices were measured and adipokine levels in serum and liver samples were detected with ELISA, Western blotting and real time fluorescence quantitative PCR (qRT-PCR).

Results: The weight, biochemical parameters and adipokine levels in serum of the model group were elevated compared to the control group ($P < 0.05$). In addition, the protein and mRNA expression levels of adipokines in the liver were significantly altered compared to the control group ($P < 0.01$). The therapy group was characterized by decreased weight and biochemical indices ($P < 0.05$) compared with the model group. Supplementing high-fat diet with omega-3 PUFA decreased serum levels of leptin and resistin, while adiponectin levels were slightly elevated. In liver tissue samples, the protein and mRNA expression levels of adipokines were significantly improved ($P < 0.01$) in the therapy group compared to the model group.

Conclusion: Omega-3 PUFA improved lipid and glycometabolism in NASH rats and regulated adipokine expression, indicating that omega-3 PUFA may have a therapeutic benefit for patients with NASH.

Key words: Adipokines, non-alcoholic steatohepatitis, polyunsaturated fatty acid

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Introduction

Obesity is an increasing problem for health care systems. In addition to development of metabolic syndrome, obesity increases the risk of developing non-alcoholic fatty liver disease (NAFLD). The incidence of non-alcoholic steatohepatitis (NASH), a severe form of NAFLD, is rising dramatically and is considered to be a risk factor for hepatic fibrosis and hepatocellular carcinoma of underlying etiology.[1] Therefore, there is a critical need to develop novel therapeutic approaches to prevent and reverse NASH. Metabolic syndrome is characterized by several key components such as type 2 diabetes, dyslipidemia, atherosclerosis and hypertension[2]. Insulin resistance (IR) has been shown to play a central role in the pathogenesis of NASH. Recent studies have also demonstrated that disturbances of lipid metabolism, increased oxidative stress, increased lipid peroxidation, and altered adipokine expression levels are associated with the pathogenesis of NASH.[2]

Adipokines, such as leptin, resistin and adiponectin, are cytokines that are predominantly secreted by the adipose tissue.[3] Recent studies have indicated that leptin, resistin and adiponectin play key roles in management of the metabolic homeostasis of the liver and modulate lipid metabolism, which facilitates fat accumulation and inflammation of liver even in the absence of obesity, dyslipidemia, and diabetes.

Polyunsaturated fatty acid (PUFA), such as omega-3 PUFA (also known as omega-3 fatty acids), are essential fatty acids for humans. Recent evidence suggests that omega-3 PUFAs have beneficial effects in the regulation of lipid metabolism, improvement in IR status and controlling blood pressure. A recent study has demonstrated that omega-3 PUFAs have the ability to reverse severe hepatic steatosis, ameliorate hepatocellular damage, lobular inflammation and other common features of NASH, and to reduce oxidative stress.[4] Therefore, PUFA has the potential for treating or preventing NASH. The goal of our study was to evaluate the effects of omega-3 PUFA on adipokine expression levels in a rat animal model of NASH.

Materials and Methods

Animal models

Thirty-two male Sprague-Dawley rats (140–160 g) were purchased from Shanghai Slac Laboratory Animal Co. Ltd of the Chinese Academy of Sciences (production license:

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SCXK(SH)2007–0005). The animals were housed in a temperature and humidity controlled room (22.0 ± 2.0°C and 65 ± 5%, respectively) with a 12-hour dark-light cycle. The animals were allowed free access to food and water, and were cared for and utilized according to the guidelines approved by the Animal Ethics Committee of Shanghai Jiaotong University. The rats were divided into three groups according to their weight after being fed a normal diet for 7 days. The control group (n = 10) was fed a standard rodent diet (from Shanghai Slac Laboratory Animal Co. Ltd). The model (n = 10) and therapy groups (n=12) were fed a high-fat and high-cholesterol diet (HFD) that consisted of 88% standard diet, 10% lard oil and 2% cholesterol (from Shanghai Slac Laboratory Animal Co. Ltd). Xu, et al., previously demonstrated that the NASH model was established after the animals were fed the HFD for a period of 8 weeks.[5] After 8 weeks, the therapy group was treated with omega-3 PUFA (1.0 g/d) by intragastric administration. The omega-3 PUFA (seal oil) was obtained from Tianjin Tasly Pharmaceutical Co. Ltd. The composition of omega-3 PUFA (per 100g) was DHA (7.2 g), EPA (6.5 g), and DPA (3.6 g). At 20 weeks, the animals were subjected to overnight fasting and anesthetized with intraperitoneal injection of 10% chloral hydrate (0.35 mL/100g body weight) prior to tissue collection. Blood samples were collected from the abdominal aorta and centrifuged at room temperature for 15 min at 3000 g. The serum was preserved at -70°C until assayed. Liver tissue was collected for paraffin embedding and liver homogenates, which were prepared according to the routine method. Snap frozen liver samples were kept frozen at -70°C for real time fluorescence quantitative PCR (qRT-PCR) and Western blotting. Visceral adipose tissue was collected and stored at -70°C for evaluation by ELISA.

Hepatic pathological evaluation

Liver tissues (4-µm sections) were stained with hematoxylin-eosin (HE) by standard methods. The pathological changes were observed under light microscopy in a blinded fashion. The degree of hepatocyte steatosis and inflammatory cell infiltration were analyzed using the NAFLD activity score (NAS). The criteria utilized were as follows: (1) for hepatic steatosis: grade 0, no fat; grade 1, steatosis occupying less than 33% of the hepatic parenchyma; grade 2, 34%–66% of the hepatic parenchyma; grade 3, more than 66% of the hepatic parenchyma; (2) for inflammatory cell infiltration: grade 0, none; grade 1, 1–2 foci/field; grade 2, 3–4 foci/field; grade 3, more than 4 foci/field; and (3) for hepatocyte ballooning: grade 0, none; grade 1, a few; grade 2, many.

Evaluation of serum biochemistry and adipokine levels

The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglyceride (TG), fasting blood glucose (FBG) and free fatty acid (FFA) were measured with an automatic biochemistry analyzer (HITACHI 7600, Japan) utilizing commercial kits (Wako Pure Chemical Industries, Ltd, Japan). Adipokine levels (resistin, leptin and adiponectin) in both serum and visceral adipose tissue were measured with ELISA-based commercial kits (R&D, Inc., USA).

Western blotting

Whole cell lysates were prepared using lysis buffer (Shanghai Bioleaf Biotech Co., Ltd). Lysates were centrifuged at 14,000 rpm for 10 min to remove insoluble material and the supernatants were resolved with a 10% SDS gel. The proteins were transferred to a nitrocellulose membrane, and the membranes were incubated with polyclonal rabbit antibodies against leptin, resistin, adiponectin (1:500, Abbiotec, LLC, USA), and then appropriate secondary antibodies. Protein bands were visualized by enhanced chemiluminescence as described in the manufacturer's protocol (Invitrogen Inc., USA). The expression of ß-actin was examined for each blot and served as the loading control.

qRT-PCR

Total RNA from frozen tissue biopsies was extracted using Trizol reagent (Promega Inc. USA) according to the manufacturer's instructions. Primers and probes for qRT-PCR used in this study were purchased from Invitrogen Inc. USA (Shanghai agency). Sequences of primers are listed in Table 1. RNAase inhibitor, dNTP and Oligo (dT) were purchased from TOYOBO Company (Tokyo, Japan). The mRNA expression levels of adipokines were adjusted relative to GAPDH. GAPDH was used as endogenous control to ensure equal starting amounts of cDNA. qRT-PCR experiments were repeated in three independent experiments.

Statistical analyses

All data were analyzed with SPSS 13.0 statistical package. Data were expressed as mean ± SD. Statistical differences between means were determined by a factorial design ANOVA, which was followed by a least-significant difference test when there was no interaction between variables. Multiple comparisons between the groups were performed using SNK method. $P < 0.05$ was considered statistically significant.

Results

Omega-3 PUFA therapy reduces body weight and liver weight in HFD-fed animals

All animals survived to the completion of the experimental period. Body and liver weights were clearly higher in the HFD-fed rats after 20 weeks. In the omega-3 PUFA-treated therapy group, both liver and body weights were significantly lower compared to the model group. The reduction in liver and body weights were proportional resulting in a similar liver index (LI, calculated as liver weight/body weight × 100%) value for the model and therapy groups. These results are summarized in Table 2.

Omega-3 PUFA therapy improves liver pathology in HFD-fed animals

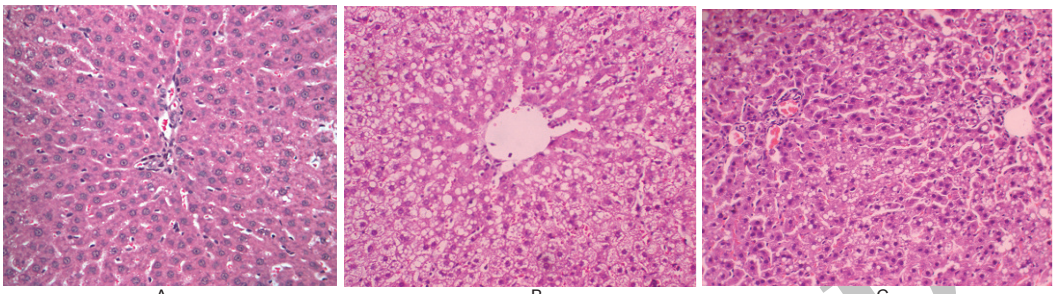
On gross examination, the control group livers were bright red with a smooth surface, had sharp edges and no adhesions to their ambient tissue. However, livers of the model group, as compared

Table 1. Real-time fluorescence quantitative PCR Primer Sequences.

Gene	Primer (forward, 5'-3')	Primer (reverse, 5'-3')	Product size
Leptin	GGTGGCTGGTTTGTTTCTGT	TATGTGGCTGCAGAGGTGAG	249bp
Resistin	CTAGCTGCTCCTGTGGCTCT	AGGGCAAGCTCA GTTCTCAA	155bp
Adiponectin	AATCCTGCCCAGTCATGAAG	TCTCCAGGA GTGCCATCTCT	159bp
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCC GTGGGTAGAGTCAT	207bp

Table 2. Effects of HFD and ω-3 PUFA on body weight and liver weight.

Group	*n*	Liver weight (g)	Body weight (g)	Liver index (g/Kg)
Control	10	10.22 ± 2.38	357.67 ± 21.24	2.84 ± 0.53
Model	10	21.01 ± 1.67①	521.43 ± 27.80①	4.04 ± 0.35
Therapy	12	15.96 ± 1.41②	434.33 ± 15.89②	3.67 ± 0.23
Compared with control group, ①P<0.01; Compared with model group, ②P<0.01				



A B C

Figure 1. HE staining of liver tissue from (A) control group, (B) model group, (C) therapy group. Original magnification: ×200.

Table 3. NAS of three groups.

Group	*n*	Steatosis	Inflammatory	Ballooning	Score
		0 1 2 3	0 1 2 3	0 1 2	
Control	10	10 0 0 0	10 0 0 0	10 0 0	0
Model	10	0 1 5 4	0 2 5 3	0 6 4	5.8 ± 0.3①
Therapy	12	0 5 4 3	0 4 6 2	3 7 2	4.5 ± 0.5②
Compared with control group, ①P < 0.01; Compared with model group, ②P < 0.01					

Table 4. Effects of HFD and ω-3 PUFA on serum biochemistry.

Group	*n*	FBG(mmol/L)	AST(U/L)	ALT(U/L)	TC(mmol/L)	TG(mmol/L)	FFA(μmol/L)
Control	10	4.56 ± 1.28	85.83 ± 15.79	41.33 ± 16.08	1.76 ± 0.68	0.28 ± 0.06	363.57 ± 35.07
Model	10	8.71 ± 1.68②	136.57 ± 20.25②	64.29 ± 22.40①	4.65 ± 2.24②	0.51 ± 0.17①	651.78 ± 87.63①
Therapy	12	7.25 ± 2.02	128.33 ± 33.07	43.83 ± 7.36③	2.77 ± 1.42③	0.38 ± 0.21	557.81 ± 63.52③
Compared with control group, ①P < 0.05, ②P < 0.01; Compared with model group, ③P<0.05							

with those of the control group, were markedly enlarged, yellow brown in color, and had blunt edges with adhesions to surrounding tissue making the liver difficult to remove. Livers from the therapy group were yellow-red in color with blunt edges. On evaluation after HE staining, hepatocytes in the control group exhibited normal pathology with no inflammatory cell infiltration or necrosis (Figure 1A). In the model group, the liver tissue was characterized by steatosis and ballooning degeneration of hepatocytes with necrosis and inflammatory cell infiltration, which were predominately monocytes with a few lymphocytes and neutrophilic granulocytes. These lesions were observed in the lobules and portal area (Figure 1B). In the therapy group, liver tissue was characterized by reduced steatosis, as compared to the model group, and less inflammatory cell infiltration without necrosis (Figure 1C). Steatosis and inflammatory infiltration are compared between the three groups in Table 3.

Omega-3 PUFA therapy improves serum biochemistry and alters adipokine levels

Compared with the control group, both ALT and TG levels were significantly elevated in the model group (P < 0.05). In addition, FBG, AST, FFA and TC were significantly elevated (P < 0.01). A significant decrease in ALT, FFA and TC was observed after 12 weeks of omega-3 PUFA administration (P < 0.05). These findings are summarized in Table 4.

The serum and visceral adipose tissue levels of leptin, resistin and adiponectin were determined by ELISA. Serum leptin and resistin levels were significantly increased in the model group compared to the control group. Administration of omega-3 PUFA induced a non-significant decrease in leptin and resistin levels compared to the model group. Serum adiponectin levels were lower in the model group compared to the control rats, while serum adiponectin levels were elevated with the administration of omega-3 PUFA. In adipose tissue, omega-3 PUFA induced a significant increase in both leptin and resistin levels and a decrease in adiponectin. These findings are presented in Table 5.

Evaluation of adipokine protein and mRNA expression levels in liver tissue

The protein expression levels of leptin and resistin were significantly elevated, whereas adiponectin levels were decreased in the model group compared to the control group (Figure 2A and B). The administration of omega-3 PUFA significantly increased adiponectin levels and lowered leptin and resistin levels to those

Table 5. Evaluation of adipokine levels in serum and adipose tissue.

Group	*n*	Leptin(ng/mL)		Resistin(ng/mL)		Adiponectin(μg/mL)	
		Serum	Tissue	Serum	Tissue	Serum	Tissue
Control	10	4.51 ± 1.77	7.60 ± 0.10	4.57 ± 1.65	103.60 ± 24.26	8.56 ± 0.74	61.01 ± 22.36
Model	10	5.94 ± 0.94②	15.41 ± 2.02②	5.73 ± 0.73①	246.61 ± 41.17②	7.36 ± 2.76①	12.44 ± 3.03②
Therapy	12	5.10 ± 0.96	10.23 ± 0.83③	5.18 ± 0.77	120.95 ± 9.50③	8.44 ± 0.95	45.62 ± 6.32③

Compared with control group, ①$P < 0.05$, ②$P < 0.01$; Compared with model group, ③$P < 0.01$

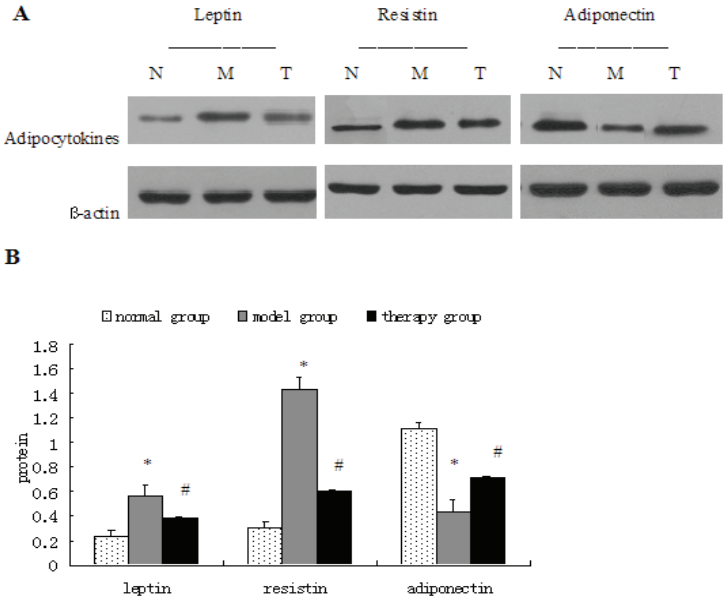


Figure 2. Adipokine protein expression in liver tissues were analyzed by Western blotting (A) from the control group (N), the model group (M), and the therapy group (T). (B) The corresponding quantification of the protein expression. Compared with the control group, * $P < 0.01$; Compared with model group, # $P < 0.01$.

Table 6. Effect of HFD and PUFA on adipokines mRNA copies (x ± s).

Group	*n*	Leptin(10^2)	Resistin(10^2)	Adiponectin(10^2)
Control	10	2.89 ± 0.23	9.02 ± 0.24	848.14 ± 154.54
Model	10	202.40 ± 65.41①	256.00 ± 19.97①	20.58 ± 5.22①
Therapy	12	38.03 ± 4.39②	65.80 ± 2.12②	269.50 ± 61.44②

Compared with control group, ①$P < 0.01$; Compared with model group, ②$P < 0.01$

similar to the control group (Figure 2A and B). The profile for mRNA expression levels of leptin, resistin and adiponectin were similar to the protein expression levels. These findings are presented in Table 6.

Discussion

With the increasing prevalence of obesity, the incidence of NAFLD is a growing issue for the healthcare systems worldwide. Sufficient epidemiological and clinical evidence showed there are mechanisms underlying the link between NAFLD and other liver diseases[2]. PUFA has been shown to regulate the fatty acid levels of phospholipids in the cell membrane, which can ameliorate endothelial function.[6] However, due to its short half-life, PUFA fails to accumulate in the human body and requires daily supplementation. Recently, interest in the role of PUFA in limiting the progression of NASH/NAFLD has become a topic of interest. Parker *et al* reported beneficial changes in liver fat accumulation and function tests of individuals treated with omega-3 PUFA in nine eligible studies.[7] In addition, PUFA displays greater effects during NASH/NAFLD with hyperlipidemia by modulating fatty acid synthesis and reducing plasma TG concentration.[8] Moreover, PUFA intake from the daily diet has been linked to improved insulin sensitivity with weight loss. In the current study, we evaluated the role of omega-3 PUFA on the modulation of liver injury and adipokine levels in a rat animal model of NASH.

A rat model of NASH was successfully generated after feeding rats HFD for 8 weeks as previously described.[5] Liver pathology indicated steatohepatitis with characteristic histological and pathological alterations. Our study confirmed that long-term HFD increased body weight and liver weight, which was associated with liver dysfunction, lipid and glucose metabolic disturbances. The

animals in the therapy group were treated with omega-3 PUFA, which improved liver function tests and other biochemical indices. In particular, there was a significant decrease in serum TC and fatty acid levels. In addition, there was a decrease in hepatic steatosis, inflammation and necrosis in the animals treated with omega-3 PUFA compared to the model group. These findings suggest that omega-3 PUFA has a beneficial effect in animals on HFD by reducing steatosis and subsequent liver damage.

Recent evidence suggests that adipokines, which are secreted by the adipose tissue, may participate in the pathogenic progression of NASH/NAFLD.[3] Leptin has been demonstrated to have profibrogenic actions both *in vitro* and *in vivo*. Long-term leptin treatment, improving steatosis grade and ballooning injury, has positive effects on NASH patients.[9] Machado, et al., found high serum leptin levels and decreasing adiponectin levels in biopsy-proven NASH patients. The progressively increasing serum leptin was associated with severity of steatosis ($P = 0.032$) and fibrosis ($P = 0.053$).[10] High serum leptin and low adiponectin levels were also observed in both NAFLD and obesity groups. In a study by Li, et al., logistic regression analysis showed a positive correlation between leptin and waist-to-hip ratio and homeostasis model assessment-insulin resistance (HOMA-IR), and an inverse association between adiponectin and HOMA-IR and body mass index (BMI).[11] They concluded that the increased serum leptin level and decreased serum adipoectin level in NAFLD patients are independently associated with HOMA-IR. Similar findings were presented by Yazici, et al.[12] Recent evidence suggests an association between adiponectin and leptin genotypes. In a study in a Chinese population, it was found that adiponectin-45 and leptin-2548 might play a role in predisposition to the progressive form of NAFLD or NASH.[13] The leptin receptor 223A>G polymorphism could be a predictive marker for macroangiopathic complications due to the predisposition to high plasma leptin levels[14]. Jiang, et al., confirmed high serum resistin and low adiponectin in NAFLD patients. Serum resistin concentration was positively correlated with waist circumference, and no correlation was found between resistin levels and blood pressure, FBG, or TC.[15] In contrast, serum adiponectin levels were negatively correlated with waist circumference, FBG and BMI.[12,15] Adiponectin is the most abundant adipose-specific adipokine that has been shown to possess multiple beneficial effects in obesity-related medical complications; it decreases hepatic and systematic IR, and attenuates liver inflammation and fibrosis.[16] Obesity is thought to contribute to the process of NAFLD and is correlated to low adiponectin levels, which has a protective role against insulin resistance and obesity-related liver diseases.[16] These previous findings indicate that serum leptin, resistin and adiponectin levels may be suitable serum markers for predicting advanced liver disease in NAFLD. In fact, adiponectin levels were found to be a surrogate marker of hepatic fibrosis and were elevated significantly in cirrhosis cases.[17] However, another study indicated that serum adiponectin levels were not associated with the severity of hepatic fibrosis.[18]

In our previous study, a similar trend of adipokine expression levels (increased leptin and resistin and decreased adiponectin) was found in the serum of animals fed HFD compared to control animals.[19] In the current study, animals treated with PUFA had decreased leptin and resistin levels in serum and adipose tissue, while adiponectin raised progressively. Relatively, the modification of adipokine expression in the visceral adipose tissue occurred with a more obvious trend than in serum. Therefore, our findings indicate that treatment with omega-3 PUFA plays a role in the down-regulation of leptin and resistin, as well as the up-regulation of adiponectin expression. Interestingly, there was not a significant change in the levels of adipokines in the serum of the therapy group compared to the model group, while significant changes were observed in both the visceral adipose and liver tissues. This finding suggests that the modifications of adipokine expression levels in the adipose tissue may precede alterations in the serum levels. However, further studies are necessary to determine whether the length of administration or the dosage levels of omega-3 PUFA alter the serum levels more dramatically than those found in the current study.

During our study, there was no evidence to indicate changes in the animals' overall condition and growth with varying treatment, suggesting that omega-3 PUFA is relatively safe for administration. From the clinical standpoint, it is important to consider the implementation of proper diet and exercise programs in addition to omega-3 PUFA administration. In conclusion, our study demonstrated that omega-3 PUFA not only improved liver inflammation, damage and metabolic disturbances, but also regulated adipokine expression in the liver and visceral adipose tissues. These findings indicate that omega-3 PUFA may be an important therapeutic tool to prevent or reverse NASH. Further studies are warranted to determine the mechanisms by which omega-3 PUFA alters adipokine expression during NAFLD and NASH.

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