Original Paper

Beneficial effects of Omega-3 polyunsaturated fatty acids in treatment of non-alcoholic fatty liver disease in rats

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is sickness with different causes and it is the most hepatic illness. The objective of this work is to investigate the bimolecular changes in a model of high fat diet induced- NAFLD in rats. Also, the hypolipidemic, antioxidant and anti-inflammatory effects of omega-3 were clarify via evaluation of some biochemical and molecular parameters in the liver tissue of rats.Twenty one adult white albino rats were divided into three groups. Group I (Normal control): received no drugs and fed balanced diet, Group II (NAFLD- induced group): rats fed high fat diet for 8 weeks followed by normal basal ration for additional 8 weeks. Group III (NAFLD + omega-3): rats administrated omega-3 (300 mg/kg b.wt/day), orally for 8 weeks post-induction of NAFLD. The obtained results showed a significant increase in serum Total cholesterol and Triacylglycerols (TAG) concentrations, L-MDA and TAC in liver tissue with significant up-regulation in CPT-1α, FAS, SREBP-1c, PPAR-α, Nrf2 and TAK1gene expression in NAFLD-induced rats. Treatment with Omega-3 to NAFLD induced rats potentially improved molecular hepatic cell function and oxidative alterations related to NAFLD near its normal ranges. Meanwhile, histo-pathological findings supported that Omeg-3 markedly attenuates harmful effects of NAFLD and protected liver cell. On conclusion, Omega-3 plays an important role as potent anti-oxidant, anti-obesity, anti-inflammatory via inhibition of hepatic steatosis and oxidative stress singling.

1. INTRODUCTION

Non-alcoholic fatty liver illness is a major medical issue because of its high predominance. NAFLD is related with obesity, insulin resistance, diabetes mellitus type 2 (DM2), hypertension, hyperlipidemia and metabolic condition. NAFLD covers a wide pathological range from steatosis to Nonalcoholic steatohepatitis (NASH) to various levels of liver fibrosis, cirrhosis and Hepatocellular carcinoma (HCC) (Berná and Romero-Gomez, 2020).

Omega-3 fatty acids (ω-3) are essential fatty acids not synthesized de novo by people. Fish oil, flaxseed and nuts are known as exceptionally rich wellsprings of ω-3. The dietary supplementation with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can managecreation of pro-inflammatory cytokines as interleukin and Tumor necrosis factor-α (TNF-α), which are secreted when macrophages and monocytes are initiated (De Oliveira et al., 2017).

Lipid accumulation in the liver occurs due to an imbalance in hepatic lipid utilization, synthesis, degradation and secretion. Accelerated lipolysis cause increase serum free fatty acids (FFAs)in the peripheral adipose tissue and visceral fat in the state of insulin resistance (Tessari et al., 2009). Consequently, the current study believes to evaluate the possible protective effect of omega-3 polyunsaturated fatty acids against the NAFLD induced following high fat regimen in adult male albino rats through evaluation of serum lipids profile, antioxidant status, oxidative stress and inflammatory mediators of some gene expression levels in addition to histopathological alterations of liver tissues.

2. MATERIAL AND METHODS

2.1. Experimental animals:

Twenty-one male albino rats, 8-10 weeks old and average body weight 160 ± 10 g was used in this study. Rats were fed on constant ration and clean drinking water was supplied ad-libitum. All rats were acclimatized for 10 days prior to the start of experiment. Animal Care and Use committee at Benha University were approved this experimental protocols and were in accordance with the National

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Institute of Health Guide for the Care and Laboratory Animals.

2.2. Chemicals and antioxidant agent:
1-Fish oil (Omega-3):
Omega-3was acquired as soft gelatin capsules, each capsule contain 1000 mg omega-3 fatty acids. Fish oil (Omega-3 plus) was made by South Egypt Drug Industries Co. (SEDICO) 6th October city, Egypt. Omega-3 was broken down in propylene glycol and taken orally. The dosage of omega-3 plus was picked to be in the therapeutic range levels at a dose of (300mg/kg/day) (Meganathan et al., 2011)

2-Propylene glycol, formaldehyde solution and normal saline:
Propylene glycol, formaldehyde solution and normal saline were purchased from ADWIC, El Nasr Pharmaceuticals Company for Trading Chemicals and Medical Appliances, Egypt

2.3. Experimental design:
Rats were acclimatizing to the laboratory conditions and partitioned into three equal groups randomly:

Group I: (Normal control group): Consisted of 7 rats, received no treatment, provided only with a constant standard pellet diet and plenty of clean water ad-libtium for 16 weeks.

Group II :(High fat diet-induced NAFLD group):Contained 7 rats received no treatment, and were fed on high fat diet (60% fat, 22% carbohydrates, 18% protein) daily for 8 weeks for induction of NAFLD, followed by ordinary basal ration feeding for another 8 weeks (Li et al., 2014).

Group III: (NAFLD + omega 3 treated group): Include 7 rats fed HFD for 8 weeks for induction of (NAFLD) by the end of the 8th week, all rats fed normal basal ration and treated orally with omega 3 fatty acid(300 mg/kg b.wt/day) (Meganathan et al., 2011)

2.4. Sampling:
2.4.1. Blood samples:
Blood samples were collected via veins puncture of the medial canthus of the eye, kept to clot, then centrifuge for 15 minutes at 3,000 rpm. Serum were separated in dry sterile tubes by automatic pipette, and then stored at -20°C in a deep freeze until use for subsequent biochemical analysis.

2.4.2. Liver for molecular analysis:
All rats were sacrificed, after 16 week of the experiment, by cervical decapitation, the abdomen was open and the liver was rapidly excised gently, cleaned by rinsing with ice-cold sodium chloride solution 0.9%, immediately kept in liquid nitrogen and stored at -80°C till RNA extraction for the real time quantitative polymerase chain reaction (qPCR) analysis of the hepatic gene expressions levels Carnitine-palmitoyltransferase-1α (CPT-1α), Fatty acid synthase (FAS), Sterol regulatory element binding protein-1c (SREBP-1c), Peroxisome proliferator-activated receptor-α(PPAR-α), Nuclear factor erythroid 2-related factor 2 (Nrf2) andTransforming growth factor-β activated kinase 1 (TAK1). Also, small part of liver tissue were put in Eppendorf tubes and kept in a deep freeze at -20°C until

use for Malondialdehyde (MDA) and total antioxidant capacity (TAC) determination.

2.4.3. Liver for histopathological examination:
Liver of rats in all groups were examined by naked eyes for any lesions after 16 weeks. Small liver specimen were taken and immediately fixed in natural buffered formalin 10% and tissue paraffin sections were routinely prepared and stain with H& E according to Bancroft and Gamble, (2008).

2.5. Analysis:
2.5.1. Biochemical analysis:
Serum triacylglycerols and total cholesterol concentrations were determined by enzymatic method described by Fossati and Prencipe (1982) and Allain et al., (1974), respectively. Moreover, liver MDA and TAC were determined according to the method of Mesbah et al., (1974) and Koracevic et al., (2004), respectively.

2.5.2. Molecular analysis:
Real-time PCR with SYBR Green was used to measure expression of mRNAs of target genes in the liver. The isolated cDNA were amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer protocol and gene specific primers. The primers used in the amplification are shown in Table A. The web based tool, Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used to design these primers based on published rat sequences.

Table (A): Forward and reverse primers sequence for real time PCR.

Gene	Forward primer (5 ------ ´3)	Reverse primer (5 ------ ´3)
Nrf2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
PPAR-a	CCTGTCCGTCGGGATGTCACAC	GTGACGGTCTCCACGGACATGC
CPT-1a	CAGCTCGCACATTACAAGGA	TGCACAAAGTTGCAGGACTC
FAS	GGATGTCAACAAGCCCAAGT	CAGAGGAGAAGGCCACAAAG
SERBP-1c	GGCCCTGTGTGTACTGGTCT	AGCATCAGAGGGAGTGAGGA
TAK1	CCATCCCAATGGCGTATCTTACA	TCATCCTGGTCCAATTCTGCAA
β-actin	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC

2.6. Statistical analysis:
The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS, version 18.0 software (2011). All the data were expressed as means ±S.E. The individual comparisons were obtained by Duncan's multiple range test (DMRT). Values were considered statistically significant when (P<0.05).

3. RESULTS
The obtained data illustrated in table (1) exhibited that serum total cholesterol and triacylglycerols concentrations were significantly increase (P≥0.01) in NAFLD group (G2) contrasted with control group (G1). Treatment of NAFLD with omega-3 (G3) displayed significant decrease in serum total cholesterol and triacylglycerols concentrations (P≤0.01) compared with NAFLD group (G2).

Table 1 Effect of treatment with Omega-3 polyunsaturated fatty acids on serum total cholesterol and triacylglycerols concentrations in NAFLD-induced rats.

Animalgroups	Total Cholesterol (mg/dl)	Triacylglycerols (mg/dl)
Normal control (G1)	88.69[d]± 4.45	43.38[d]± 2.34
NAFLD (G2)	177.25[a]± 8.92	99.42[a]± 5.72
NAFLD treated with omega-3 (G3)	137.12[c]± 6.71	70.40[b]± 3.62

Data are presented as Mean ± SEM (Standard error of mean).
Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

The results presented in Table (2) revealed a significant increase (P≤0.05) of Liver tissue L-malondialdehyde (L-MDA) concentration in NAFLD rats (G2). This raised was significantly decreased following administration of omega-3 (G3).Meanwhile, a significant decrease in Total antioxidant capacity (TAC) was observed in NAFLD-induced rats as compared to control group (G1). Conversely, TAC levels of omega-3 treated group (G3) showed significant increase as compared to NAFLD group (G2).

Table 2 Effect of treatment with Omega-3 polyunsaturated fatty acidson liverMDA and TAC concentrations in NAFLD-induced rats.

Animal groups	MDA (nmole/g. tissue)	TAC (mmole/g. tissue)
Normal control (G1)	$33.14^d \pm 1.54$	$3.94^a \pm 0.21$
NAFLD (G2)	$69.50^a \pm 3.58$	$1.5^d \pm 0.08$
NAFLD treated with omega-3 (G3)	$51.23^b \pm 2.60$	$2.40^c \pm 0.13$

Data are presented as Mean ± SEM (Standard error of mean).
Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

The obtained results presented in table (3) revealed a significant up regulation (P≤0.05) of CPT-1α and FAS gene expression level in liver of NAFLD rats (G2) as compared with control group (G1). However, the expression levels of the CPT-1α remained significantly higher in omega-3 treated group (G3) and liver FAS gene expression was down regulated after omega-3 treatment compared with the NAFLD rats (G2).

Table 3 Effects of Omega-3 polyunsaturated fatty acids treatment on the relative expression ofCPT-1α and FAS gene in liver tissues of NAFLD-induced rats.

Animal groups	Fold change (Relative quantification) Mean ± SEM	
	CPT-1α	FAS
Normal control (G1)	$1.00^c \pm 0.1$	$1.00^c \pm 0.07$
NAFLD (G2)	$5.21^b \pm 0.27$	$9.65^a \pm 0.53$
NAFLD treated with omega-3(G3)	$9.51^a \pm 0.48$	$4.06^c \pm 0.22$

Means within the same column carrying different superscript letters are significantly different (P≤ 0.05).

The results illustrated in Table (4) revealed a significant up-regulation (P≤0.05) of SREBT-1, PPAR-αand TAK1 gene expression level in liver of NAFLD rats (G2) when compared with normal control group (G1). This elevated expression of SREBT-1 and TAK1 were significantly down regulated, however in PPAR-α gene stay significantly higher following administration of omega-3 (G3). Additionally, liver tissue NrF2 gene expression level revealed a significant (P≤0.05) down regulation in NAFLD rats (G2) followed by significant up-regulation after omega-3 treatment (G3) as compared with (G2).

Table (4): Effects of Omega-3 polyunsaturated fatty acids treatment on the relative expression of SREBT-1, Nrf2, PPAR-α andTAK1 gene in liver tissues of NAFLD-induced rats.

Animal groups	Fold change (Relative quantification) Mean ± SEM			
	SREBT-1	NrF2	PPAR-α	TAK1
Normal control (G1)	$1.00^d \pm 0.08$	$1.00^d \pm 0.09$	$1.00^e \pm 0.08$	$1.00^c \pm 0.10$
NAFLD (G2)	$3.10^a \pm 0.2$	$0.92^d \pm 0.08$	$2.75^d \pm 0.16$	$3.43^a \pm 0.28$
NAFLD treated with omega-3(G3)	$2.36^b \pm 0.14$	$1.82^c \pm 0.11$	$5.24^b \pm 0.28$	$2.22^b \pm 0.09$

Means within the same column carrying different superscript letters are significantly different (P≤ 0.05).

Histopathological examination

Group I: (normal control group fed on normal ration):
The microscopic examination of (H&E) stained liver sections from rats of control group revealed normal hepatic architecture. Hepatocytes are arranged in trabecules running radiantly from the central vein and are separated by irregular blood sinusoids. Also contains portal tract consisted of portal vein, hepatic artery and bile duct (Fig.1).

Group II: (Nonalcoholic fatty liver disease group):
The microscopic examination of (H&E)-stained liver sections from rats of NAFLD group, supplemented with HFD daily for 8 weeks, showed marked degree of hepatic steatosis and hydropic degeneration of hepatocytes (Fig 2) congestion of central vein and portal blood vessels was seen. Also, focal area of necrosis was also detected among hepatic parenchyma which presented by pyknotic nuclei with more esinophilic hepatic cytoplasm..

Group III: (Nonalcoholic fatty liver disease treated with omega-3):
The microscopic examination of (H&E) stained liver sections in this group administrated omega 3 showed moderate degenerative changes of hepatocytes in the form of hydropic (ballooning) degeneration of hepatocytes (Figs. 3).
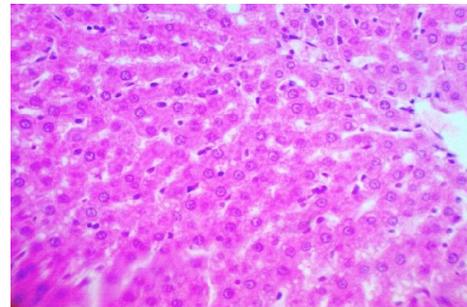

Figure (1): Hematoxylin and eosin (H&E)-stained liver section of a rat from normal control group showing hepatocytes arranged in trabeculae running radially from the central vein and separated by sinusoids ×400 .
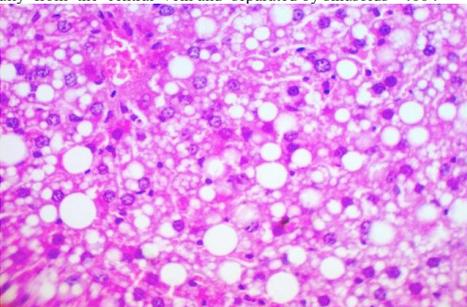

Figure (2): Hematoxylin and eosin (H&E) stained liver section of a rat from NAFLD group, showing marked degree of hepatic steatosis and hydropic degeneration of hepatocytes × 400).
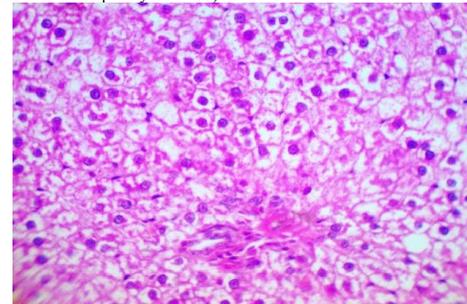

Figure (3): Hematoxylin and eosin (H&E)-stained liver section of a rat from NAFLD treated with Omega 3 polyunsaturated fatty acids, showing degeneration of hepatocytes x400.

4. DISCUSSION

The existing results exhibited a significant increase in serum total cholesterol (TC) and triacylglycerols (TAG) concentrations in NAFLD induced rats compared with control group. Treatment of NAFLD with omega-3 displayed significant decrease in serum total cholesterol and triacylglycerols concentrations compared with NAFLD group. Similarly, Parker et al. (2012) showed that ω-3 together diminished the measure of liver fat. Another meta-examination meant to survey the impact of ω-3 on liver fat and blood lipid levels in patients with NAFLD and NASH. The consequences demonstrated that ω-3 can streamline liver fat and HDL levels in patients with NAFLD, proposing the helpful capability of ω-3 in liver sickness (lu et al., 2016). Also, omega-3 unsaturated fat supplementation has been appeared to diminish irritation, upgrade insulin affectability, and improve hyper-triglyceridemia (Alwayn et al., 2005). Omega -3 have also been used to effectively improve dyslipidemia (Tanaka et al., 2008). Administration of ω-3 decrease plasma TAG which might be related with glycemic control as demonstrated in an investigation of NASH patients with diabetes (Dasarathy et al., 2015). Treatment with omega-3 reversed all biochemical and genetic dys-regulation seen in HFD-fed rats. There was a decrease in blood glucose, TAG, and TC indicating lowering fat load from blood (Rodriguez-Ramiro et al., 2016), and this in accordance with the current finding. Moreover, Toshimitsu et al., (2007) stated that individuals with NAFLD have been shown to have a lower dietary intake of omega-3 fatty acids than healthy controls. The obtained data showed a significant increase of Liver tissue L-malondialdehyde (L-MDA) concentration in NAFLD rats. This raised was significantly decreased following administration of omega-3when compared with NAFLD untreated group. Likewise, Zelber-Sagi et al. (2020) stated that serum MDA is strongly associated with NAFLD and markers of NASH or fibrosis among men. Dietary vitamin E intake seems to be protective from elevated MDA levels. However, the role of ω-3 on MDA plasma levels remains unclear. Some trials observed that after ω-3, MDA plasma levels decreased (Véricel et al., 2015).Meanwhile, a significant decrease in Total antioxidant capacity (TAC) was observed in NAFLD-induced rats as compared to control group (G1). Conversely, TAC levels of omega-3 treated group showed significant increase as compared to NAFLD group. The obtained data are nearly similar with those reported by (Makni et al. 2008) who showed that dietary intake of fish oil lead to significant improvement in antioxidant status and oxidative stress markers with elevation in total antioxidant capacity (TAC) levels, while malondildehydes (MDA) level were significantly reduced. Omega-3 has displayed cyto-protection against lipid peroxidation. Moreover, Fish oil has modulatory effects on antioxidant status (TAC, MDA) as they elevates total antioxidant capacity and decrease lipid peroxidation (MDA). Besides they showed significant hepato-protective power confirmed by attenuation of liver functions and modulation of hepatic histology in hyper-cholesterolemic male rats (Farahat et al., 2017).Additionally, latest researches demonstrated that genes involving oxidative stress and mitochondrial dysfunction weredown-regulated in treated group, and this was in line with the changes of hepatic antioxidant enzyme activities (Li et al., 2017).

The obtained results revealed a significant up regulation (P≤0.05) of CPT-1α and FAS gene expression level in liver of NAFLD-induced in rats as compared with control.

However, the expression levels of the CPT-1α remained significantly higher in omega-3 treated group (G3) and liver FAS gene expression was down regulated after omega-3 treatment compared with the NAFLD rats (G2).Besides the de novo lipogenesis, fish oil feeding regulated TAG and expression of genes involved in fatty acids beta-oxidation. These imply that fish oil can promote hepatic fatty acids beta-oxidation and TAG excretion, contribute to the amelioration of liver steatosis (Yuan et al., 2016).

A significant up-regulation of SREBT-1, PPAR-α and TAK1 gene expression level in liver of NAFLD rats when compared with normal control group. This elevated expression of SREBT-1 and TAK1 were significantly down regulated, however in PPAR-α gene stay significantly higher following administration of omega-3. An animal fact has shown that this livergene associated with a pro-inflammatory state and increased lipogenesis leading to steatosis (Leclercq et al., 2011). Conversely, omega-3 are known to down-regulate sterol regulatory element binding protein 1c (SREBP-1c) and up-regulate peroxisome proliferator activated receptor a (PPAR-a) which would favor fatty acid oxidation and reduce steatosis (Pettinelli et al., 2009).It has been demonstrated that omega-3 activate the peroxisome proliferator-activated receptor (PPARα), which in turn stimulates fatty acid oxidation (Zuniga et al., 2011), inhibits hepatic lipogenesis, and reduces hepatic reactive oxygen species (Ishii et al., 2009). Besides, patients with NAFLD have been shown to have a greater deficiency of omega-3 in the diet than healthy controls (Toshimitsu et al., 2007).Conversely, omega-3 are known to down-regulate sterol regulatory element binding protein 1c (SREBP-1c) and upregulate peroxisome proliferator activated receptor a (PPAR-α) which would favour fatty acid oxidation and reduce steatosis (Pettinelli et al., 2009). Dietary omega-3 also stimulate FAO by binding directly to the peroxisome proliferator activated receptor-α, (PPAR-α), i.e., a fatty acid-regulated nuclear receptor. PPAR-α regulates gene expression by binding promoters of target genes in association with the retinoid X (Pawar and Jump, 2003).In the non-canonical pathway, activated TGFβ receptors regulate EGF receptor signaling that activates RAS/ MapK, TGF 3β-activated kinase (TAk1), PI3K and Akt pathways leading to increased expression of proteins involved in fibrosis (Nyati et al., 2015).Furthermore, liver tissue NrF2 gene expression level revealed a significant down regulation in NAFLD rats followed by significant up-regulation after omega-3 treatment.

5. CONCLUSION

We can conclude that, Omega-3 polyunsaturated fatty acids has potent hepatoprotective and anti-inflammatory role against non alcoholic fatty liver induced disease, liver steatosis, oxidative stress, and inflammation by modulation of SREBP-1c, PPAR-α, Nrf2 and TAK1 and control liver lipid disorder through CPT-1α and FAS signaling.

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