Journal of Innovations in Pharmaceutical and Biological Sciences (JIPBS)
ISSN: 2349-2759
Available online at www.jipbs.com

Research article

Effects of Nigella Sativa oil and Docosahexaenoic acid on experimentally-induced hepatic fibrosis in rats

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Key words: Nigella Sativa; Docosahexaenoic acid; Liver fibrosis; PPAR-γ; CD34.

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Vol. 6 (3), 24-29, Jul-Sep, 2019.

Abstract

Hepatic fibrosis is a consequence of chronic liver injury. Peroxisome proliferator activated receptor-gamma (PPAR-γ) may play a role in the pathogenesis of hepatic fibrosis. Activation of endothelial progenitor cell (EPC) mobilization can contribute to hepatic regeneration after fibrotic injury. However, the potential benefits of docosahexaenoic acid (DHA) and Nigella Sativa oil (NS), naturally-derived oil, in ameliorating fibrosis remain elusive. Liver fibrosis was induced in rats by oral administration of 50% CCl_4 three times per week for 8 weeks. The rats intoxicated with CCl_4 were divided into three groups: fibrosis control, DHA, and NS groups. A fourth group of normal healthy rats served as a control group. The results showed that Nigella Sativa oil significantly decreased serum albumin, alanine aminotransferase (ALT), and bilirubin levels in the NS-treated group compared to those in the fibrosis control group. Liver histopathology and EPC immunohistochemistry supported these biochemical data. A significant increase in PPAR- γ was found in the DHA and NS groups. The results indicate that the NS oil treatment produced a more substantial effect than DHA treatment and has a beneficial role in inhibiting or reversing liver fibrosis.

Introduction

Hepatic fibrosis is a reversible injury healing process resulting from prolonged non-resolving inflammation. It is characterized by the unusual generation of extracellular matrix (ECM), which directs the accumulation of fibrous scar tissue and the development of irreversible cirrhosis [1]. Fibrosis following chronic hepatic injury can progress to cirrhosis and increase the risk of developing liver cancer [2]. Consequently, antifibrotics are needed to inhibit fibrogenesis from progressing to cirrhosis or to provoke regression of advanced liver fibrosis. Hepatic stellate cells (HSCs) are the principal cells involved in the process of fibrogenesis and are bound to the increased accumulation of ECM proteins in response to sustained liver injury. These cells are excited by a variety of chronic liver lesions that lead to their conversion into activated myofibroblasts, producing excess ECM deposition and the development of a fibrous scar [3].

Endothelial progenitor cells (EPCs) are essentially located within the stem cell niche in bone marrow, along with some shifting populations in the peripheral blood. EPCs have been used as curative agents for neovascularization under disease conditions. The ultimate purposes of EPC therapy are healing the lining of damaged blood vessels and producing clinical improvement with fewer adverse effects [4].

Mechanisms implicated in endogenous EPC-associated therapy include the mobilization of EPCs from the bone marrow, cytokine-guided homing with consequent engraftment into the ischaemic area, and finally, trans differentiation into endothelial cells that constitute the vasculature. The efficacy of EPC therapy in regenerative medicine depends on adequate recruitment of available cells (either exogenously supplied populations or endogenously gathered residents) to the target tissue [5].

Peroxisome proliferator-activated receptor (PPAR)-γ, an essential fatty acid-activated nuclear receptor, has a crucial role in human metabolic pathology, such as dyslipidaemia and insulin resistance [6]. PPAR-γ also plays a critical role in the control of the immune response-associated with fibrosis. PPAR-γ agonists have been shown to alleviate murine hepatic fibrosis, which is associated with the induction of TNF-α and IL-6 expression and a decline in TGF-β1 and α-SMA expression in liver tissue [7]. In the CCL_4-induced rat liver fibrosis model, infection with a recombinant lentiviral expression vector loaded with the rat PPAR-

γ gene suppressed HSC proliferation and hepatic fibrosis and decreased the expression of α-SMA and type I collagen[8].

This research aimed to investigate the effects of docosahexaenoic acid and Nigella Sativa oil on experimentally-induced hepatic fibrosis in rats.

Methods

Study design

The study included approximately sixty adult male albino rats weighing 180–230 g each, obtained from the animal house of Giza Institute of Ophthalmology, Cairo, Egypt. Rats were weighed and housed in wire mesh cages for one week under the same environmental conditions for adaptation and allowed free access to water and a standard pellet diet. The experimental protocol was approved by the local ethical committee of the Faculty of Pharmacy, Tanta University. Hepatic fibrosis was provoked by oral administration of 50% CCl_4 solution in corn oil at a dose of 0.1 mL/100 g three times weekly for 8 weeks [5]. Hepatic fibrosis was induced in forty-five male albino rats, which were then randomly arranged into three subgroups (n = 15 for each group), including a fibrosis control group and two groups treated with either docosahexaenoic acid (eBioChem. Co., China) (0.1 g/kg, orally) [5] or Nigella Sativa oil (Haraz Co., Egypt) (1 g/kg, orally) [9]. The fourth group of fifteen rats served as the normal control and received the vehicle. Docosahexaenoic acid and Nigella Sativa were administered to rats once daily by oral gavage for 8 weeks, parallel with CCl_4 administration. At the end of the experiment, rats were anaesthetized by diethyl ether (El-Nasr Pharmaceutical, Qalyubia, Egypt); blood was thencollected from the inferior vena cava and centrifuged at 3000 rpm for 10 min, and the serum was saved at -20°C until assessment of alanine aminotransferase (ALT) activity, albumin and total bilirubin concentration. After blood collection, rats were sacrificed, and liver samples were carefully excised and washed with saline. Portions of the livers were fixed in 10% formalin and then embedded in paraffin for immunohistochemical staining for CD34 and histopathological examination. The remaining liver samples were kept frozen at -80°C until the assessment of peroxisome proliferator-activated receptor gamma (PPAR-γ).

Serum biochemical measurements

Serum ALT activity, albumin and bilirubin concentrations were measured using kits obtained from Biodiagnostic (Cairo, Egypt). ELISA kits were used for the determination of PPAR-γ (Shanghai Sunred Biological Technology Co., China) according to the instructions given by the manufacturer.

Liver histopathological examination

The paraffin blocks were sectioned into 5 ml pieces, mounted on slides, and stained with haematoxylin and eosin (H&E) for histopathological examination. The sections were examined with an electric light microscope. Histological changes were evaluated by a pathologist who was unaware of the type of treatment. Liver fibrosis was investigated in each sample from the fibrosis control group according to the METAVIR system [10]. The two groups treated with either docosahexaenoic acid or Nigella Sativa oil were evaluated for regression of fibrosis, known as hepatic repair complex, which includes the following features: perforated delicate septa, isolated thick collagen fibres (not visibly attached to portal tracts, venules or septa), delicate periportal fibrous spikes, hepatic vein remnants with prolapsed hepatocytes (hepatocytes within lumens of hepatic veins), hepatocytes within portal tracts or split septa (clusters or cords of hepatocytes identified within portal tracts or trapped in fibrous septa), minute regenerative nodules, and aberrant parenchymal veins (within a 5-hepatocyte diameter from portal tracts) [11]. Then, fibrosis was classified as "predominantly progressive", "indeterminate" or "predominantly regressive" fibrosis [12].

Immunohistochemical staining FORCD34

The paraffin blocks were deparaffinized in xylene, dehydrated in a graded alcohol series, and incubated in a solution of 3% hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase. Tissue sections were incubated with primary antibody for CD34 (Catalogue no. AF4117, R&D Systems, USA). Antigen-antibody complexes were visualized by labelling with streptavidin-biotin followed by diaminobenzidine as a chromogen [13]. Slides were counterstained with haematoxylin. The examination was performed using Image Analyzer (Olympus, USA), where membranous brown expression of CD34 was evaluated in EPCs in the liver tissue in ten fields per slide.

Statistical analysis

All statistical analyses were performed using Microsoft Office Excel 2007. Differences among groups were statistically analysed by one-way analysis of variance (ANOVA). The results were expressed as the mean± standard deviation (SD). P values less than 0.05 were considered indicative of statistical significance.

Results

Serum Biochemical Parameters

As shown in table 1, the fibrosis control group showed a significant increase in serum albumin concentration ($P < 0.01$, an increase of 59%), serum alanine amino transferase (ALT) activity ($P < 0.05$, an increase of 369.3%), and serum bilirubin level ($P < 0.05$, an increase

of 134.2%) compared to the normal control group. Compared with the fibrosis control group, the groups treated with DHA (DHA; $P < 0.05$, a decrease of 24.3%) and NS (NS; $P < 0.01$, a decrease of 30.7%) exhibited a significant decrease in serum albumin. The rat group treated with NS oil also showed a significant decrease in serum ALT activity compared with the fibrosis control group; $P < 0.01$, a decrease of 64.6%. Moreover, a significant decrease in serum bilirubin was observed in the DHA group ($P < 0.05$, a decrease of 83.1%) compared to that in the fibrosis control group.

Hepatic peroxisome proliferator-activated receptor GAMMA

As shown in figure 1, the fibrosis control group showed a significant decrease in PPAR-γ content in liver tissue homogenate ($P < 0.05$, a decrease of 19.3%) compared to the normal control group. The DHA and NS oil-treated groups showed a significant increase ($P < 0.05$) in hepatic PPAR-γ content of approximately 77.7% and 43%, respectively, compared to the fibrosis control group.

Histological findings

All cases in the normal control group showed normal hepatic tissue with the central vein and extending cords of normal hepatocytes (Figure 2, A). After oral administration of a 50% CCl_4 solution, all samples from the fibrosis control group showed predominant progressive fibrosis, revealed by the histological features of cirrhosis in the form of replacement of liver tissue with regeneration nodules separated by broad fibrous septa as seen in figure 2, B. The DHA group showed indeterminate fibrosis, revealing a balance between progressive and regressive fibrosis (Figure 2, C). However, the NS oil group showed predominantly regressive fibrosis with remnant portal tracts (Figure 2, D).

Immunohistochemical findings

Positive membranous expression of CD34 was observed only in the NS oil group, and expression was negative in the other groups (normal control, fibrosis control and DHA groups) (Figure 3). Semiquantitative assessment of histological and immunohistochemical findings within all studied groups is shown in table 2.

Table 1. Effect of docosahexaenoic acid and Nigella Sativa oil on different serum biochemical parameters.

Group	Serum Albumin (g/dL)	ALT (U/L)	Serum Total Bilirubin (mg/dL)
Normal Control	4.3±0.22	55.4±27.7	0.38±0.33
Fibrosis Control	6.84±0.36[a]	260.2±50.43[a]	0.89±0.89[a]
DHA Treated	5.18±1.17[b]	175±65.23	0.15±0.14[b]
NS Treated	4.74±0.21[b]	92.2±68.13[b]	0.38±0.23

ALT, alanine amino transferase; DHA, docosahexaenoic acid; NS, Nigella Sativa. Data are presented as the mean ± SD.
a: significant *versus* normal control; b: significant *versus* fibrosis control. Significance was set at $P < 0.05$.

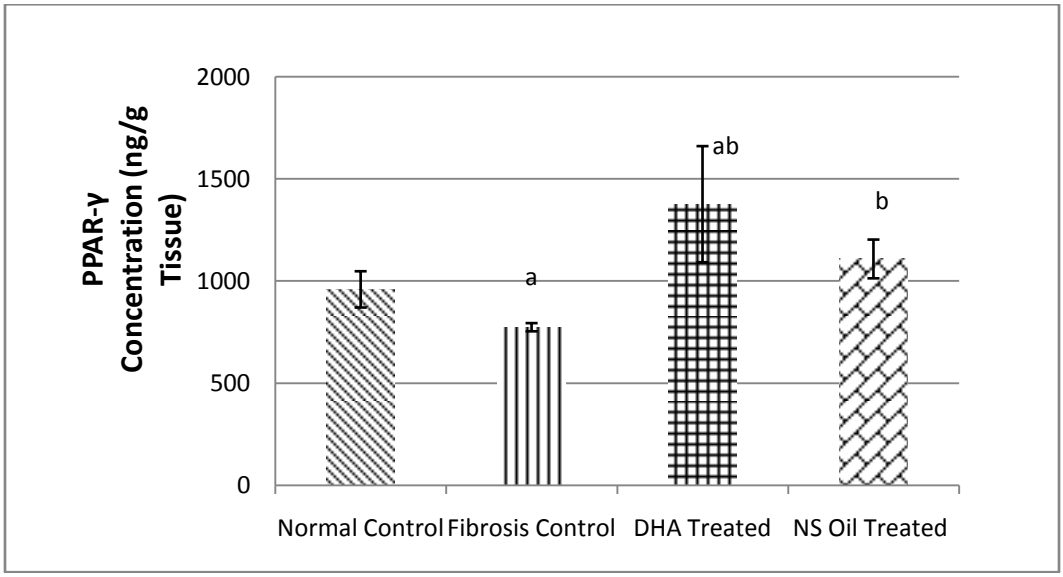

Figure 1. Effect of DHA and NS oil onhepatic PPAR-γ content. PPAR-γ, peroxisome proliferator-activated receptor gamma; DHA, docosahexaenoic acid; NS, Nigella Sativa. Data are presented as the mean ± SD. a, significant *versus* normal control; b, significant *versus* fibrosis control. Significance was set at $P < 0.05$.

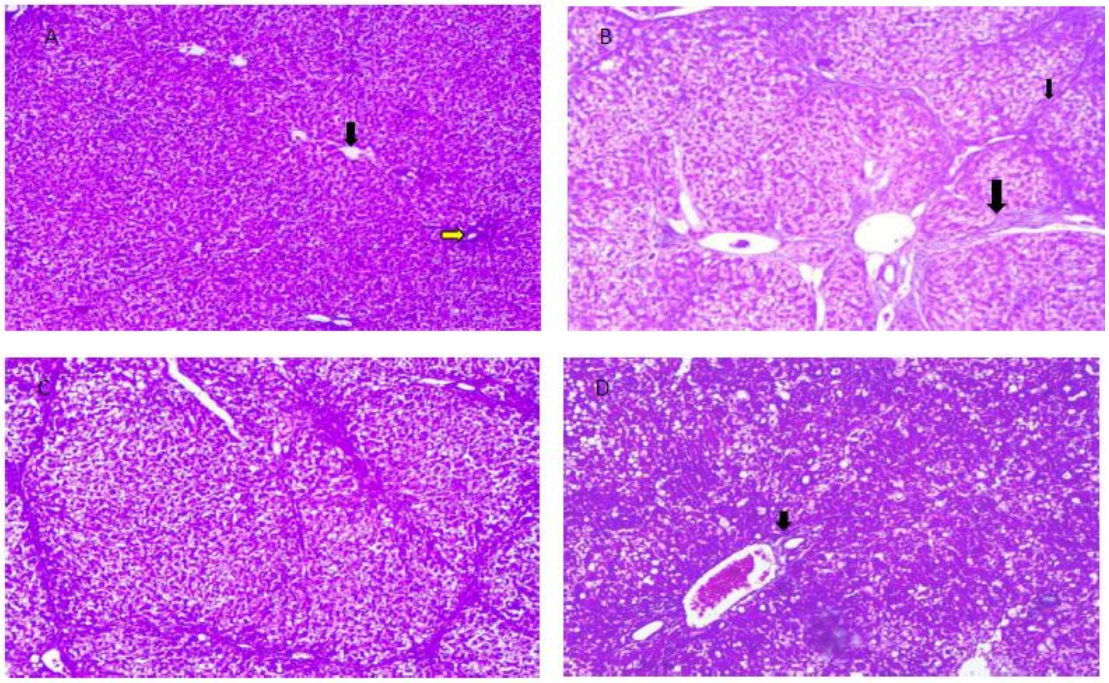

Figure 2. Histopathological comparison of liver sections stained with H&E (x40) among the studied groups. (A) Normal control group showing preserved hepatic lobule. Each lobule is formed by acentral vein (black arrow) surrounded by cords of normal hepatocytes and portal tract at the periphery (yellow arrow); (B) Fibrosis control group showing replacement of normal liver tissue with multiple fibrotic nodules surrounded by fibrous septa (black arrow); (C) DHA group showing indeterminate fibrosis in the form of balance between progressive and regressive fibrosis; (D) NS oil group showing predominantly regressive fibrosis exhibiting a marked reduction in fibrous septa with remnant portal tracts (black arrow).

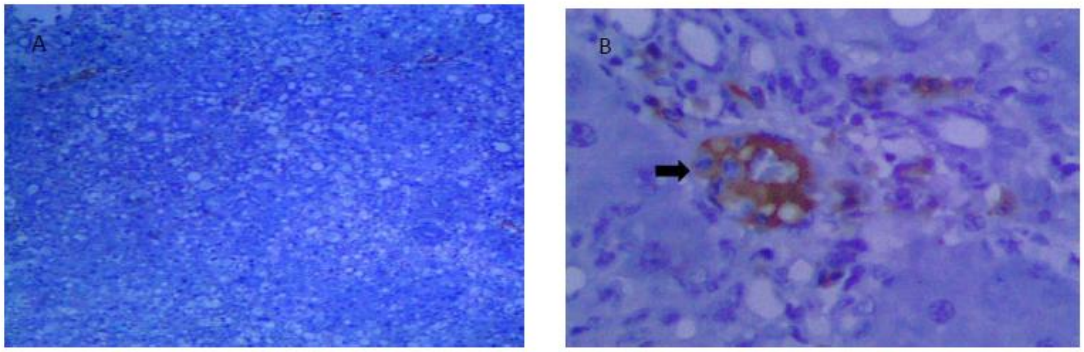

Figure 3. Immunohistochemical evaluation of liver tissue sections stained with CD34. (A) Normal control group showing negative immunohistochemical expression of CD34+ EPCs; (B) Positive membranous and cytoplasmic expression of CD34+ EPCs around the portal tract (black arrow) (IHC x200); EPCs, endothelial progenitor cells.

Table 2. Assessment of hepatic histological and immunohistochemical findings within all studied groups.

Groups	Predominant progressive fibrosis	Indeterminate fibrosis	Predominant regressive fibrosis	CD34
Normal control	-	-	-	-
Fibrosis control	+	-	-	-
DHA	-	+	-	-
NS oil	-	-	+	+

DHA: Docosahexaenoic acid, NS: Nigella Sativa, +: Present, -: Not detected.

Discussion

Liver fibrosis is a complex inflammatory and fibrogenic process, and the progression of fibrosis leads to cirrhosis. The only therapeutic remedies are the elimination of injurious stimuli and liver transplantation. Therefore, the development of anti-fibrotic therapies is desirable [14]. In this study, we examined the anti-fibrotic effects of DHA and Nigella Sativa oil monotherapy on rats with fibrosis.

In the present study, we chose CCl_4 to induce liver fibrosis in rats. Liver fibrosis can be induced by differentapproaches.CCl_4causeschemical changes to the membrane permeability in liver mitochondria and plasma in various animals and forms highly toxic free radicals, most likely mediated by cytochrome p450 2E1b [15]. A single injection of CCl_4 results in acute liver damage that leads to hepatocyte necrosis, while repeated and prolonged treatment (≥4 weeks) causes severe liver disease such as fibrosis and cirrhosis [16]. In liver fibrosis, the accretion of extracellular matrix is a marked feature, and initiation of HSCs is the antecedent of this phenomenon. In the typical liver, HSCs are dormant and not fibrogenic [17]; however, the cells are triggered by inflammatory processes involved in liver damage, such as the secretion of toxic cytokines and recruitment of inflammatory cells [18].

Many researchers have used liver enzymes as useful hallmarks of CCl_4 hepatotoxicity [19]. CCl_4 intoxication is characterized by a significant increase in the biochemical markers of liver injury (ALT, AST, ALP, and gamma-glutamyl transferase (GGT)). Increases in these enzymes are attributed to hepatic structural damage because they are localized to the cytoplasm and released into the circulation after cellular and mitochondrial damage [20]. In the current study, hepatic fibrosis was manifested by multiple fibrotic nodules in the fibrosis control group. These histological observations were associated with significantly elevated ALT activity and serum bilirubin with a decline in serum albumin. Administration of either DHA or NS oil as monotherapy decreased the fibrosis scores, but the greatest decrease was found in NS oil-treated rats and was marked by the reduction in fibrous septa with remnant portal tracts. These treatments also improved the synthetic albumin function of the liver and partially decreased the levels of bilirubin as well as ALT activity.

The results of this research demonstrated the protective valueof Nigella Sativa oil and DHA via the PPAR-γ pathway in regressing hepatic fibrosis in a rat model. Decreased hepatic PPAR-γ levels are reported to be associated with HSC activation and consequently fibrotic progression [21]. The results of this work showed that hepatic PPAR-γ content in the fibrosis control group was decreased compared to that in the normal control group, strengthening the evidence for liver fibrosis.

The present results can be confirmed by reports indicating that the alleviated inflammation and ECM accumulation in liver fibrosis were pertinent to PPAR-γ activation [22].

Our results are in accordance with the work of [23], who demonstrated that thymoquinone, a bioactive constituent of Nigella Sativa, prevented the characteristic features of induced metabolic syndrome in rats that might have been mediated *via* the PPAR-γ mechanism [24] and demonstrated that DHA enhanced the upregulation of PPAR-γ and protected the liver from induced chemical hepatic injury.

Only treatment with NS oil caused an increase in CD34+EPCs homing to liver tissue with improved vascularization, which was supported by immunohistochemistry. Such results may be accompanied by the beneficial effect of NS oil treatment in the prevention of liver fibrosis, although both treatments increased the level of hepatic PPAR-γ.

EPCs restore hepatic functional parameters to normal levels by regulating hepatocyte regeneration and ameliorating established liver fibrosis [25].

Conclusion

Treatment with Nigella Sativa oil restores inflammation-induced liver fibrosis possibly through affecting progenitor cell recruitment. Administration of Nigella Sativa oil can seemingly be considered a part of liver fibrosis management.

Declaration of interest

The authors declare that no ambivalence of interest could be perceived as prejudicing the impartiality of the research reported.

Funding

This work did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

Acknowledgements

Not applicable.

Author contribution

All authors contributed equally to this work.

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