Effect of diet and omega-3 fatty acids in NAFLD

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Abstract
Nonalcoholic fatty liver disease (NAFLD) ranges from simple hepatic steatosis to steatohepatitis (NASH) and cirrhosis. The aim of this study is to test beneficial effects of omega-3 fatty acids (DHA 130 mg, EPA 25 mg) treatment in NAFLD, in a mouse model of non-alcoholic fatty liver disease. As pretreatment, 50 mice were fed for one month with a high-fat diet to induce NAFLD. Then, the mice were divided into different groups according to diet (normo- or hypercaloric), with and without treatment with omega-3 fatty acids, for another month, forming the post-treatment group. The liver and blood samples were collected for biochemical and histopathological analysis. Biochemical parameters including: glycemia, total cholesterol, triglycerides, uric acid, albumin, total plasma antioxidant capacity (TEAC) was measured in serum. Glutathione (GSH), total thiols and malondialdehyde (MDA) were determined in mouse liver homogenates. Mice from post-treatment group, on hypercaloric diet with or without omega-3 fatty acids treatment, had medium hepatopathy (granular and vacuolar degeneration of the hepatocytes) and hyperglycemia. Omega-3 fatty acid treatment lowered the rise of triglycerides (p<0.05), glycemia (p<0.01) and cholesterol (p<0.02) in serum and MDA level of the liver (p<0.05). Mice from post-treatment group, on normocaloric diet with or without omega-3 fatty acid had different histopathological and biochemical results. Those with normocaloric/normolipidic diet and omega-3 fatty acids treatment had reversed liver histopathological results from NASH to normal aspect and had the best metabolic parameters results. In conclusion, omega-3 fatty acids treatment associated with a normocaloric/normolipidic diet has hepatoprotective action in nonalcoholic fatty liver disease.

Keywords: NAFLD, omega-3 fatty acids, oxidative stress, diet, mice.

Introduction
Non-alcoholic fatty liver disease (NAFLD) has been recognized as a major health burden and as the most important cause of chronic liver disease. NAFLD is characterized by hepatocyte triacylglycerol accumulation (steatosis), which can progress to inflammation, fibrosis, and cirrhosis (steatohepatitis) [1].

Increased lipogenesis, together with hyperlipidemia and increased fat deposition, contribute to nonalcoholic fatty liver disease [2]. Hyperglycemia and hyperinsulinemia induce lipogenesis, thereby increasing the hepatic pool of fatty acids. This pool is also increased by increased delivery of fatty acids through the diet or lipolysis in adipose tissue [3].

It was demonstrated that hepatic steatosis was alleviated by omega-3 polyunsaturated fatty acids (PUFAs) [3–6]. Dietary intake of omega-3 PUFAs had insulin-sensitizing actions in adipose tissue and liver and improved insulin tolerance in obese mice. Genes involved in insulin sensitivity: peroxisome proliferator activated receptor-γ (PPAR-γ), glucose transporter (GLUT-2/GLUT-4), and insulin receptor signaling (IRS-1/IRS-2) were up-regulated by omega-3 PUFAs [4].

It was demonstrated that omega-3 PUFAs decrease muscle intramyofibrillar triglycerides and liver steatosis. This last effect results on the one hand, from a decreased expression of lipogenesis enzymes and of delta-9 desaturase via a depleting effect on sterol response element binding protein-1c (SREBP-1c) [7]. On the other hand, n-3 long chain-polyunsaturated fatty acids (LC-PUFA) stimulate fatty acid oxidation in the liver via the activation of peroxisome proliferator activated receptor-α (PPAR-α) [7].

In sucrose-induced obese rats, consumption of dietary fish oil had beneficial effects because changes in the n-3 PUFAs composition in hepatic and adipose tissues alter membrane properties and modify the type of substrates available for the production of active lipid metabolites acting on insulin resistance and obesity [5].

The protective effects of omega-3 fatty acids are attributed not only to eicosanoid inhibition but also to the formation of novel biologically active lipid mediators: resolvins and protectins (with insulin-sensitizing, anti-inflammatory, and adiponectin inducer effects) [4].

In high fat diet fed mice increased hepatic steatosis, hyperglycemia and DNA damage were attributed not only to eicosanoid inhibition but also to the formation of novel biologically active lipid mediators: resolvins and protectins (with insulin-sensitizing, anti-inflammatory, and adiponectin inducer effects) [4].

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This study intended to assess the effects of omega-3 fatty acids on the parameters of oxidative stress, lipid metabolism and glycemic control in a high-fat diet mouse model of non-alcoholic fatty liver disease.

Materials and Methods

Animals

Male mice NMRI (18–20 g) purchased from Animal Facility of „Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania, were used through this study. The experimental procedures were carried out under Convention 86/609/E.E.C. from November 24, 1986, for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes. The animals were treated with free access to drinking water and feed under constant room temperature (25°C) and humidity (50±10%) with an automatic 12 hours light/dark cycle.

Group N, control group, was formed by ten mice that were fed a standard mouse chow diet. The pretreatment group was formed by mice that were fed a high fat diet (80% lipids, chocolate ad libitum) for one month. Each week the mice were weighed to checkup if they took on weight and at the end of the month, they had 45 g. From the pretreatment group, ten mice (Group A) were immediately sacrificed. The other mice were divided in different groups according to diet (normo- or hypercaloric), with and without treatment with omega-3 fatty acids (387 mg/day, Queisser Pharma – Doppelherz), for another one month. Each week the mice were weighed to checkup if they took on weight and at the end of the month, they had 45 g. From the pretreatment group, ten mice (Group A) were immediately sacrificing. The other mice were divided in different groups according to diet (normo- or hypercaloric), with and without treatment with omega-3 fatty acids; Group S, mice were fed along with a standard diet; Group F, mice were fed along with a standard diet; Group E, mice were fed along with a high fat diet; Group D, mice were fed along with a standard diet and received treatment with omega-3 fatty acids; Group B, mice were fed along with a high fat diet; Group F, mice were fed along with a standard diet; Group S, mice were fed along with a standard diet and received treatment with statins 0.1 mg/g/day.

At the end of the post-treatment month, ten mice from each experimental group were sacrificed by cervical dislocation and their livers were excised and immediately placed in an ice bath. Approximately equal portions of each liver were blotted on filter paper and weighed on a pharmaceutical balance (Shimadzu AY220). The pooled liver samples were homogenized in KCl 0.15 M, 10% pharmaceutical balance (Shimadzu AY220). The pooled liver samples were homogenized in KCl 0.15 M, 10% dislocation and their livers were excised and immediately placed in an ice bath. Approximately equal portions of each liver were blotted on filter paper and weighed on a pharmaceutical balance (Shimadzu AY220). The pooled liver samples were homogenized in KCl 0.15 M, 10% dilution at 200°C for glutathione (GSH) assay, in EDTA (Fluka Analytical) 0.02 M, pH 4.7, at 20°C, 5% dilution, for total thiols and in KCl 0.15 M, 25% dilution, at 20°C, for lipid peroxides assay. A Potter–Elvehjem instrument was used as a tissue homogenizer. The resultant homogenates were centrifuged 15000 g for 30 minutes at 40°C in a centrifuge (Heraeus S-R). The supernant fluid from each homogenate was carefully collected and maintained in an ice bath until used. The protein content was determined by adding 1 mL of a 1:4 dilution of the supernatant fluid to 4 mL of biuret reagent. The protein content was established by relating the absorbance to a standard curve based on bovine albumin (Sigma Chemical Co., St. Louis, MO, USA), at 550 nm.

Determination of GSH

Liver GSH and total thiols content were measured by a colorimetric technique, based on the production of a yellow color when 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma Chemical Co.) was added to compounds with sulfhydryl groups. The absorbance was read at 412 nm and calculation was done using molar extinction coefficient (adapted method of Beutler E et al.) [9].

Estimation of MDA

The quantitative measurement of lipid peroxidation in liver homogenate was determined according to the method described by Esterbauer H and Cheeseman KH [10]. The amount of MDA formed was quantified by reaction with Thiobarbituric Acid (TBA, Sigma T-5500) and the pink colored complex was measured at the wavelength 532 nm. The blood samples were collected from carotid artery and serum was separated by centrifugation at 3000 g for 10 minutes and kept at -7°C until use. The assay for: glycemia, total cholesterol, triglycerides, uric acid, albumin was done by kits (Hospitex Diagnostics, Romania). Total plasma antioxidant capacity was evaluated by the ABTS decolorization assay (TEAC). The standard curve was done with Trolox and the results are expressed in mmol/L Trolox [11]. ABTS, 2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), potassium persulphate, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma Chemical Co.

Histopathological studies

Following autopsy, livers were immediately removed and fixed in 10% formalin for at least 24 hours. The paraffin-embedded samples were then prepared and cut into 5-µm-thick sections using a rotary microtome. The sections were stained with Hematoxylin–Eosin dye and mounted with Canada balsam. The histopathological slides were examined and photographs were taken using Carl Zeiss Jena amplual type photomicroscope.

Results

Biochemical plasma results (metabolic parameters and plasma total antioxidant capacity) are present in Table 1.

| Table 1 – The values for the measured plasma parameters |
|---------------------------------|---|---|---|---|---|---|---|
| **Group** | **N** | **Group A** | **Group E** | **Group B** | **Group D** | **Group F** | **Group S** |
| **Glucose [mg/dL]** | 151.94<sup>a</sup> | 138.51<sup>b</sup> | 222.33 | 160.25<sup>c</sup> | 160.42<sup>d</sup> | 189.76<sup>e</sup> | 155.58<sup>f</sup> |
| **Cholesterol [mg/dL]** | 154.37<sup>a</sup> | 182.53 | 210.52 | 167.31<sup>c</sup> | 147.12<sup>d</sup> | 168.42<sup>c</sup> | 150.27<sup>c</sup> |
| **Triglycerides [mg/dL]** | 98.21<sup>a</sup> | 156.36 | 193.55 | 133.08<sup>d</sup> | 127.62<sup>e</sup> | 165.83<sup>c</sup> | 190.8<sup>f</sup> |
| **Albumin [g/dL]** | 3.72 | 4.04 | 3.5 | 3.18 | 3.4 | 3.43 | 3.99 |
| **Uric acid [mg/dL]** | 2.79<sup>a</sup> | 3.85 | 5.24 | 6.06 | 3.77<sup>c</sup> | 3.4<sup>d</sup> | 2.84<sup>e</sup> |
| **Total proteins [g/dL]** | 8.1 | 8.92 | 7.35 | 8.53 | 7.98 | 7.07 | 7.9 |
| **TEAC [mM Trolox]** | 1.52 | 1.5 | 1.45 | 1.48 | 1.47 | 1.4 | 1.54 |

Student t-test, comparison of each group with Group E:<sup>1</sup><sup>p<0.05</sup>,<sup>2</sup><sup>p<0.04</sup>,<sup>3</sup><sup>p<0.03</sup>,<sup>4</sup><sup>p<0.02</sup>,<sup>5</sup><sup>p<0.01</sup>,<sup>6</sup><sup>p<0.005</sup>.
The most modified metabolic parameters were in Group E (obese mice that were kept on a high fat diet). The values for plasma glucose, cholesterol and triglycerides were much higher compared to control mice (fed a standard diet). In the post-treatment group, omega-3 fatty acids treatment and statin treatment lowered the increased values of these metabolic parameters. The best results in lowering cholesterol and triglycerides were obtained in the Group D (mice with normocaloric/normolipidemic diet associated with omega-3 fatty acids).

Oxidative stress markers measured on liver homogenates are present in Table 2.

### Table 2 – The values for the oxidative stress markers measured on liver homogenates

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH [μmol/g tissue]</th>
<th>Total thiols [μmol/g tissue]</th>
<th>MDA [nmol/g tissue]</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10.2</td>
<td>16.44</td>
<td>20.95</td>
</tr>
<tr>
<td>A</td>
<td>4.2</td>
<td>11.21</td>
<td>29.51</td>
</tr>
<tr>
<td>E</td>
<td>4.42</td>
<td>10.91</td>
<td>26.3</td>
</tr>
<tr>
<td>B</td>
<td>6.8</td>
<td>9.89</td>
<td>25.77</td>
</tr>
<tr>
<td>D</td>
<td>7.25</td>
<td>10.73</td>
<td>34.6</td>
</tr>
<tr>
<td>F</td>
<td>4.99</td>
<td>5.52</td>
<td>25.7</td>
</tr>
<tr>
<td>S</td>
<td>4.2</td>
<td>5.36</td>
<td>34.6</td>
</tr>
</tbody>
</table>

Student t-test, comparison of each group with Group E 1(\(p<0.05\)), 2(\(p<0.04\)).

In Group E (obese mice that were kept on a high fat diet), the values for liver glutathione (GSH), total thiols and malonyldialdehide (MDA) were significantly modified compared to control and reflect an increased oxidative stress (low GSH and low total thiols and high-lipid peroxidation-MDA). In the post-treatment group, omega-3 fatty acids treatment and statin treatment reduced the increase in liver MDA. In the post-treatment obese group, mice without omega-3 treatment (with standard diet) and those on statins had the lowest liver levels of total thiols, concentrations lower that those in obese mice kept on a high fat diet.

### Histological results

Following microscopic examination of the liver samples and histological grading of the hepatopathy, the images reveal (Figure 1):

- **Group A** – hepatocyte swelling, sometimes associated with karyomegaly, granular or finely vacuolated cytoplasm, numerous activated Kupffer cells, indistinct sinusoids (no visible lumen), because of the volume increase of the hepatocytes, unaltered portal fields. These lesions are consistent with a medium hepatopathy (granular and vacuolar degeneration of the hepatocytes).
- **Group B** – diffuse hepatocyte swelling and volume...
In our study, following microscopic examination of the liver samples and histological grading of the hepatopathy, medium hepatopathy was described in mice fed a high fat diet for one month (Group A, obese, pretreatment group). In post-treatment group, both groups of obese mice that were kept on a high fat diet (with and without omega-3 fatty acids treatment – Groups E and B) still had medium hepatopathy (granular and vacuolar degeneration of the hepatocytes). The best histological result (normal liver architecture) was obtained in obese mice who were fed a normal diet (normocaloric/normolipidic) associated with omega-3 fatty acids (Group D) for one month.

Currently, antioxidants are of great interest due to the described association between obesity, cardiovascular alterations and oxidative stress. Also, high monounsaturated and polyunsaturated fatty acids diets are being also considered due to their potential benefits on hypertension, insulin resistance and triglyceride levels [15].

Many studies reported that omega-3 polyunsaturated fatty acids (omega-3 PUFAs) have lipid-lowering effects in animal models and human studies [14, 16–18]. The improvement in lipid profile is due mainly to enhanced fatty acid beta-oxidation and suppression of fatty acid synthesis in the liver by the PUFAs [19].

An important effect of omega-3 PUFA is the protective effect against high fat diet induced insulin resistance. They prevent the decrease of phosphatidyl-inositol-3'-kinase (PI3 kinase) activity and the depletion of the glucose transporter protein GLUT4 in the muscle. Also, they inhibit both the activity and expression of liver glucose-6-phosphatase which could explain the protective effect with respect to the excessive hepatic glucose output induced by a high fat diet [7].

Repletion of liver n-3 LCPUFA (long-chain polyunsaturated fatty acids) levels by n-3 LC PUFA dietary supplementation in high fat diet obese mice reduces hepatic lipid content, with concomitant antioxidant and anti-inflammatory responses favoring insulin sensitivity [20].

In a randomized, double blind, placebo-controlled trial, done in patients with type 2 diabetes with insulin resistance, α-lipoic acid, omega-3 fatty acids and vitamin E treatment showed the improvement in insulin sensitivity [21].

In our study, the highest values for plasma cholesterol, triglycerides and glycemia were observed in obese high fat diet mice, who continued the high fat diet for a month. These differences in metabolic serum variables between groups were not associated with differences in histopathological aspects. Groups B, E and F, all had medium severe hepatopathy. Our result is in agreement with a clinical study documented on liver biopsy done in thirty-nine patients. The researchers demonstrated that the severity of the histological changes that characterize NAFLD could not be assessed using serological parameters [22].

Dyslipidemia and insulin resistance are important culprits in developing NAFLD [2, 3]. Many clinical studies demonstrated that all subjects with NAFLD had dyslipidemia [22, 23] and that glycemia is correlated with histological activity grade [22, 24, 25]. Therefore, improving serum lipid profile and serum glucose level may be important for NAFLD prognosis. In our study, in all treated groups with omega-3 fatty acids, significant plasma lowered levels for these variables were obtained. The best metabolic parameters were obtained in Group D (with normocaloric/normolipidic diet associated with omega-3 fatty acids).

Oxidative stress and hepatic mitochondria play a role in the pathogenesis of nonalcoholic fatty liver disease [26]. Omega-3 PUFAs have a potential protective role against ROS-induced oxidative cellular damage in rat organs, especially in the liver. The attenuation of hepatic fibrosis by EPA (eicosapentaenoic acid) was significantly related to hepatic ROS levels. EPA also suppressed increases...
in hepatic ROS levels and reduced serum oxidative markers, such as 8-isoprostanone and ferritin [12]. A moderate calorie-restricted cod-based diet was found as a useful strategy to lose weight, which was accompanied by a specific improvement on oxidative stress markers [27].

Glutathione is a tripeptide that scavenges toxic metabolites, and has found in many mammalian tissues (especially in liver), and plays a very important role in the antioxidant defense system [28]. Reduced and total liver glutathione contents were diminished in mice with NAFLD [20, 26].

Repletion of liver n-3 LCPUFA levels by n-3 LCPUFA dietary supplementation in high fat diet obese mice reduces hepatic lipid content, with concomitant antioxidant and anti-inflammatory responses favoring insulin sensitivity [29]. MDA is one of the products of oxidative stress formed during lipid peroxidation process. Hepatic lipoperoxide concentrations were significantly increased in Wistar rats with liver steatosis due to a choline-deficient diet [26].

In this study, we estimated the liver oxidative stress, measuring, on the one hand, the liver antioxidants (GSH, total thiol and TEAC) and on the other hand, the oxidative stress marker MDA. In blood, albumin, uric acid and TEAC are important antioxidants. In the obese mice, fed a high fat diet, the liver glutathione was reduced with 56.66% and liver total thiol were reduced with 33.6% compared to normal (Group E vs. Group N), while liver MDA was increased significantly (p<0.01). In the post-treatment group, in the obese mice, omega-3 fatty acids treatment lowered the rise of liver lipid peroxidation (p<0.05) and improved the liver glutathione and total thiol concentrations but no statistical significant values were observed for these antioxidants. Knowing that oxidative stress is involved in steatohepatitis development [26] and because treatment reduced the liver oxidative stress, we can assert that omega-3 fatty acids can prevent advanced stages in NAFLD.

Conclusions
Both animal models and human intervention trials, showed a beneficial effect of n-3 PUFAs on the severity of NAFLD as expressed by laboratory parameters and imaging measurements. In our study, diet (normocaloric/ normolipidic) associated with omega-3 fatty acids can reverse medium hepatopathy to normal liver architecture, reduce plasma cholesterol, plasma triglycerides, fasting plasma glucose and liver lipid peroxidation in obese (high fat diet) mouse model of non-alcoholic fatty liver disease. This study emphasizes the importance of the association between standard diet and omega-3 fatty acids not only to prevent progression of NAFLD to advanced stages, but also to reverse histopathological modifications of NAFLD to almost normal liver architecture.

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