EXPERIMENTAL STUDY

Effects of fish oil on cell proliferation and liver injury in an experimental model of acute hepatic injury induced by carbon tetrachloride

Korkmaz H1, Temel T2, Bugdaci MS3, Tekelioglu Y4, Ozoran Y5, Kapicioglu S6

Selcuk University, Faculty of Medicine, Department of Internal Medicine Division of Gastroenterohepatology, Selcuklu, Konya, Turkey. husein68@hotmail.com

Abstract: Objective: We aimed to investigate the effect of fish oil on the hepatic injury and cell cycle phases as well as cellular proliferation-regeneration in a rat model of acute hepatic injury induced by carbon tetrachloride. Background: Compensatory cell proliferation and tissue regeneration occurs as an endogenous response following chemical damage to the liver and enable animals to over come the injury. Data related to effect of fish oil on liver injury induced by chemical hepatotoxins are controversial. Method: 60 male Wistar-albino rats were fed either with a diet supplemented with 20% fish oil or standard rat feed for 2 weeks. After an overnight fast, rats in each group were administered either 1 ml/kg carbon tetrachloride or saline intraperitoneally. Results: Fish oil enriched diet significantly enhanced the carbon tetrachloride-associated necroinflammatory damage, ballooning degeneration and the elevation of serum transaminases induced by carbon tetrachloride. Furthermore, fish oil diet prevented cell proliferation, increased the proportion of cells in the G0/G1 phase concurrent with a decrease in the proportion of cells in the S phase cells. Conclusion: Fish oil diet exacerbates the hepatic injury and prevents cell proliferation-regeneration in normal and injured liver cells. Suppression of tissue regeneration by fish oil may lead to progression of the hepatic injury (Tab. 3, Fig. 4, Ref. 31). Text in PDF www.elis.sk.

Key words: hepatic injury, fish oil, n-3 PUFAs, cell-cycle, carbon tetrachloride.

Carbon tetrachloride (CC14) is a classic hepatotoxicant, which causes acute and reversible liver injury characterized by centrilobular necrosis followed by hepatic regeneration and tissue repair (1). CC14 induced hepatotoxicity involves two phases. The first phase is cytochrome P-450 (Cyt P-450) mediated metabolism of toxic trichloromethyl free radicals (e.g. CC13, CC13O2) which initiates lipid peroxidation and leads to peroxidative degradation of cellular membranes leading to the necrosis of hepatocytes (1, 2). The second phase is the activation of Kupffer and sinusoidal endothelial cells probably by free radicals and release of proinflammatory and cytotoxic mediators like tumour necrosis factor α (TNF-α), interleukin 1 (IL-1) and eicosonoids (2–4). Those inflammatory mediators from activated hepatic macrophages are thought to potentiate CC14 induced hepatic damage (3, 4).

Fish oil contains high amounts of omega-3 polyunsaturated fatty acids (n-3 PUFA) including eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), which are essential fatty acids. Dietary n-3 PUFAs may decrease concentration of n-6 PUFAs including arachidonic acid (ARA) in the membranes of all cell types particularly platelets, monocytes and liver cells. ARA products are proinflammatory mediators (2-series eicosanoids) whereas EPA products (3-series eicosanoids) have antiinflammatory effects. The competitive inhibitory effects of ARA and EPA on cyclooxygenase and lipooxygenase enzymes may explain this opposite inflammatory effects (5, 6). In addition, n-3 PUFAs also decrease TNF-α and IL-1 production from Kupffer cells and macrophages (7, 8).

Dietary interventions rich in EPA and/or DHA have been shown to keep inflammation under control and therefore are used as preventive measures against a number of illnesses such as rheumatoid arthritis, ulcerative colitis, asthma and cardiovascular diseases (9). Whereas some papers describe undesirable effects of n-3 PUFAs (10, 11). Supplemeting the diet with n-3 PUFAs resulted in an increased lipid peroxidation (12). Dietary n-3 PUFAs have been shown to have a detrimental effect on wound healing (10). It has been also reported that fish oil rich in the PUFAs accelerate lipid peroxidation induced by CC14 but do not enhance CC14 induced liver injury (13). In contrast, Polavarapu et al (14) showed...
that fish oil caused severe liver damage in alcohol-intoxicated rats. Therefore, the effect of fish oil on liver injury induced by chemical hepatotoxins is controversial.

Generally, n-3 PUFAs such as EPA and DHA inhibit cell proliferation in comparison to saturated fatty acid. However, some studies have shown either no impact or a positive effect of PUFAs on cell proliferation (15, 16). Based on these studies, higher concentrations of dietary PUFAs may inhibit cell proliferation in both rat and human population (17, 18). However, no information about the effect of n-3 PUFAs on cell proliferation in chemical induced hepatotoxicity is available.

The aim of this study was to evaluate the effects of dietary supplements enriched with n-3 PUFAs in the form of fish oil on hepatoprotective actions in an experimental model of CC14 induced liver injury and we aimed to determine the effect of fish oil on cell cycle phases as well as cellular proliferation-regeneration in this experimental study.

Materials and methods

Animals and Diets

Male Wistar–albino rats (from Animal Research Laboratory of Uludag University Faculty of Medicine Bursa/Turkey) weighing 125–175 gr underwent experiments performed in accordance with the guidelines for animal research of the National Institute of Health and approved by our ethic committee on Animal Research. The animals were housed individually in air conditioned rooms (21±1 °C) with controlled humidity (50±10 %) and a 12 hours (h) light/dark cycle, and were fed experimental diets for a 2-wk period. Sixty rats were divided randomly into the groups and fed either (1) a diet supplemented by 20 % (w/w) Menhaden oil FO (Menhaden FO – Sigma-Aldrich Chemie Gmbh, Munich, Germany) or (2) standard rat feed (SRF; Taris Chow Co., Izmir Turkey). Vitamin E or other antioxidants were not given to the groups supplemented with fish oil. The diets were stored at –20 °C and provided fresh daily. The major fatty acid composition of Menhaden FO and SRF diet was analyzed by gas chromatography (Tab.1). On the 16 th day, both groups (FO or control) were subdivided into the two subgroups and after a 16 h fast, either 1 ml/kg CC14 dissolved in corn oil 1:1 (from Merck KgaA, 64271 Darmstadt, Germany) or saline injected intraperitoneally (ip) injected. Four experimental groups were labelled as follows; (1) Control (SRF-saline), (2) CC14 (SRF-CC14), (3) Fish oil (FO-saline), (4) FO-CC14. Each group consisted of fifteen animals. 24 h after the administration of CC14, tail venous blood was taken for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurement. The rats were sacrificed under diethyl ether anesthesia. The liver of each rat was excised for histopathologic and cell cycle phases analysis as well as cellular proliferation-regeneration index using flow cytometry.

Assessment of liver function

The blood samples were centrifuged (Beckman GS-6R, Germany) at 4 °C for 15 minutes to separate serum. Serum ALT and AST activities were determined as international unit per litre (IU/L) by a Hitachi 747i automatic analyzer (Hitachi, Tokyo) using commercial kits.

Histological analysis

Liver samples fixed in 10 % buffered formalin were embedded in paraffin, sliced into 5 μm sections, and stained with hematoxylin-eosin for histological assessment by a registered pathologist unaware of the treatments. The histopathological changes were examined under the microscope (Nikon, Eclipse, TS100, Japan).

Cell – cycle phases analysis and cell proliferation–regeneration assay by flow cytometry

Fresh tissue was disintegrated mechanically at 4 °C in a plastic Petri dish which contains medium (RPIM, 1640 + 5 % fetal calf serum). Cell suspension was diluted by a filter with 80 μM sized pores and suspended at ice containing 0.1 % triton x – 100 PBS for 3 minutes. After ablation, cell suspension was incubated at 37 °C with RNAz for 20 minutes (180 U/ml PBS). Then stored in the dark for 1 hour at 4 °C with propidiumiodide (50 μg/ml in PBS) and flow cytometric analysis (FCA) were performed with Epics Elite EST (Coult - USA).

G0 / G1, S, G2 / M phases of the cells and DNA distribution of the cell cycle fractions were calculated with DNA polipoidi index Multi Cycle DNA computer program. (Phoenix flow systems, Inc. San Diego). These values were interpreted as the proliferative - regenerative index (PI). PI was expressed as percentage fraction of the division of sum of S phase and rate between G2 and M phases with sum of rate between G0 and G1 phases, S phase and rate between G2 and M phases. PI = (S+G2/M) + (G0/ G1+S+G2/M) X100 (19).

Statistical analysis

Statistical analysis of the results was performed using the Mann–Whitney U-test, analysis of variance (ANOVA) and the Student’s t-test. The results are expressed as the mean ± SEM and a pP value <0.05 was considered significant.

Results

Biochemical results

The serum ALT and AST levels of the study groups are presented in the Table 2. No significant difference at AST – ALT levels of control and FO groups were found. AST – ALT levels of CC14 and FO - CC14 groups were significantly higher than in the control

<table>
<thead>
<tr>
<th>Fat composition (%)</th>
<th>Menhaden fish oil</th>
<th>Standard rat feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic (C18:2 w6)</td>
<td>1.5</td>
<td>20</td>
</tr>
<tr>
<td>a-Linoleic (18:3 w3)</td>
<td>1.6</td>
<td>2</td>
</tr>
<tr>
<td>Arachidonic acid (20:4 w6)</td>
<td>0.9</td>
<td>–</td>
</tr>
<tr>
<td>EPA (20:5 w3)</td>
<td>15.5</td>
<td>–</td>
</tr>
<tr>
<td>DHA (22:6 w3)</td>
<td>9.1</td>
<td>–</td>
</tr>
<tr>
<td>Oleic (18:1 w9)</td>
<td>11.4</td>
<td>33</td>
</tr>
<tr>
<td>Palmitic (C16:0)</td>
<td>17.1</td>
<td>30</td>
</tr>
<tr>
<td>Stearic (C18:0)</td>
<td>2.8</td>
<td>13</td>
</tr>
</tbody>
</table>
and FO groups (p<0.01, 0.01). In addition AST – ALT levels of FO- CCl4 was significantly higher than in CCl4 group (p<0.01).

Histological analysis

In the control and FO group, no changes were observed in the hepatocytes, portal triad, sinusoidal structures, central vein, and hexagonal liver lobules. Almost no ballooning degenerations were observed (Figs 1 and 2).

All rats given CCl4 or FO-CC14 showed histopathological signs of acute hepatitis, reflected by necrosis, inflammatory cell infiltrate, ballooning degeneration and hemorrhage. Liver damage and histological changes were found to be significantly worse in the FO-CC14 group than CCl4 group (Figs 3 and 4).

Cell – cycle phases and cell proliferation–regeneration analysis of liver by flow cytometry

Liver cell-cycle phases and proliferation analysis of experimental groups are presented in the Table 3. Despite an increase in the proportion of the synthesize phase (S) cells and proliferation index (PI) and a decrease in the proportion of G0/G1 phase cells in the CCl4 group, the differences were not statistically significant when compared to the control group (p>0.05). When FO group was compared to the control group; percentage of cells in S phase and PI was significantly decreased (p<0.001, 0.001 respectively) and the rate of G0/G1 phase cells was significantly increased (p<0.0001). In comparison of fish oil and CCl4 groups; PI and the percentage of cells in S phase was significantly decreased (p<0.001, 0.001 respectively) and rate of G0/G1 phase cells was significantly increased (p<0.001 respectively). When FO-CC14 group was compared to the control group; the rate of S and G2 phase cells and PI was significantly decreased (p<0.001, 0.001, 0.001 respectively) and the rate of G0/G1 phase cells was significantly decreased (p<0.01, 0.01, 0.01 respectively).

Tab. 2. Serum levels of alanine and aspartate aminotransferases of study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (SRF+Saline)</td>
<td>132.67 ± 11.96</td>
<td>36.8 ± 4.48</td>
</tr>
<tr>
<td>CCl4 (SRF+ CCl4 )</td>
<td>363.14 ± 22.14</td>
<td>241.20 ± 5.82</td>
</tr>
<tr>
<td>Fish Oil (SRF+%20 Fish oil)</td>
<td>128.60 ± 10.60</td>
<td>33.47 ± 4.27</td>
</tr>
<tr>
<td>Fish oil – CCl4 (SRF+%20 Fish oil+CCl4)</td>
<td>531.13 ± 28.07</td>
<td>412.40 ± 32.98</td>
</tr>
</tbody>
</table>

*p< 0.01; **p< 0.01 as compared with control and fish oil group; ***p< 0.01 as compared with CCl4 group; SRF: Standard rat feed. Values are given as the mean ± SEM.
increased (p<0.001). In the comparison of FO-CC14 and CCl4 groups; the rate of S and G2 phase cells and PI was significantly reduced (p<0.001, 0.001, 0.001 respectively) and the proportion of cells in GI phase was significantly increased (p<0.001). When FO-CCl4 group was compared with fish oil group; the rate of S and G2 phase cells and PI was significantly decreased (p<0.002, 0.001, 0.001 respectively) and the proportion of cells in GI phase was significantly increased (p<0.001).

**Discussion**

During the past decade, dietary n-3 PUFAs have been the subject of numerous investigations. Despite some beneficial effects of n-3 PUFAs, several adverse effects are also documented (11, 12, 20).

In our study, dietary fish oil alone did not lead to any biochemical and histopathological changes in rat livers. All rats given CCl4 or Fish oil plus CCl4 showed biochemical and histopathological signs of acute hepatitis. Data in our study also showed that fish oil diet increased the elevation of serum transaminase and the liver injury induced by CCl4. Similarly Nanji et al (21) and Polavarapu et al (14) found that Omega-3 fatty acids alleviated acute oxidative damage as well as necroinflammatory changes. Kurulay et al (22) also showed that fish oil increased hepatotoxic effects of acetaminophen. In contrast, Schmöcker et al. demonstrated that n-3 PUFA alleviated D-galactosamine/lipopoly saccharide-induced acute hepatitis by suppression of cytokines (7). Further Periz et al reported that DHA blunted liver injury induced by CCl4 (23).

Multiple cellular mechanisms have been proposed to explain the effects of n-3 PUFAs, including the inhibition of ARA-derived eicosanoid biosynthesis, influences on transcription factors and gene expression, modification of signal transduction pathway and enhancement of lipid peroxidation (24, 25).

Compensatory cell proliferation and tissue regeneration occurs as an endogenous response following either partial hepatectomy or chemical damage to the liver and enable animals to overcome injury and survive (26). In this study, CCl4 group showed that CCl4 enhanced PI and cells proliferation by accelerating the G1 to S progression at 24 hours. In the study performed by Rao et al (27), in which the rats were treated with CCl4, the authors investigated that following administration of CCl4, the peak S-phase synthesis was observed at 36 h, indicating maximum DNA synthesis occurred at this time point. Our results also showed that fish oil prevented cell proliferation in Fish oil or Fish oil plus CCl4 administered rats, increased the proportion of cells in the G0/G1 phase and decreased the proportion of cells in the S phase suggesting that Fish oil in rich n-3 PUFAs prevented cell proliferation by decelerating the G1 to S progression.

Similarly, several in vitro studies have demonstrated that fish oil diet rich in n-3 PUFAs inhibited proliferation of normal and malignant cells (17, 18, 28). In the in vitro study by Noorden et al (29), the fish oil diet has been found to reduce the compensatory growth after partial surgical hepatectomy in parallel to increased lipid peroxidation. Arend et al (30) also demonstrated that a diet enriched with fish oil enhanced lipidperoxidation and suppressed reparative regeneration of the rat liver connective tissue. Recently, Chung Chiang et al (31) showed that fish oil caused inhibition of hepatoblastoma cell proliferation. In contrast, some studies have shown that PUFAs promoted cell proliferation (15, 16). At present, the exactly reason is not known. However, these differences between the experimental studies may result from a different experimental set up, for example, species used, sex and age of the animal, the period and dose of experimental treatment.

It should be noted that one of the limitations of the present study is the lack of data on lipid peroxidation antioxidant status and inflammatory mediators (TNF-α, IL-1β, IFN-γ, nitric oxide), regarding the measurements of antioxidants such as superoxide dismutase, glutathione and malondialdehyde of thiobarbituric acid reactive substances. Our results can be interpreted that fish oil can slow down cell renewal or, by deepening cell damage, it may emerge a decreased proliferation index and the synthesis phase.

In conclusion, in the present CCl4-induced acute hepatic injury model of rats, fish oil diet exacerbated the hepatic injury and prevented cell proliferation-regeneration in normal and injured liver cells. Suppression of tissue regeneration by fish oil may lead to the progression of hepatic injury.

**References**


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**Tab. 3. Liver cell cycle phases and proliferation analysis of experimental groups by flow cytometry.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CC14</th>
<th>FO</th>
<th>FO+CC14</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1 (%)</td>
<td>69.91±0.94</td>
<td>66.82±0.92</td>
<td>77.27±0.96</td>
<td>85.34±1.13abc</td>
</tr>
<tr>
<td>S (%)</td>
<td>21.07±0.79</td>
<td>23.65±0.84</td>
<td>13.42±0.61</td>
<td>9.26±0.93abc</td>
</tr>
<tr>
<td>G2/M (%)</td>
<td>9.02±0.50</td>
<td>8.53±0.27</td>
<td>19.71±0.52</td>
<td>5.42±0.42abc</td>
</tr>
<tr>
<td>PI</td>
<td>30.09±1.71</td>
<td>32.18±0.92</td>
<td>22.59±0.96</td>
<td>14.67±1.13abc</td>
</tr>
</tbody>
</table>

*p<0.05, as compared with control group; **p<0.05, as compared with fish oil group; ***p<0.05, as compared with CCl4 group. Values are given as the mean ± SEM.


Received October 28, 2012, Accepted October 27, 2013.