PREVENTION OF CARBON TETRACHLORIDE-INDUCED LIVER INJURY IN RATS BY OMEGA-3 FATTY ACIDS

OMUR KARACA SAYGILI[1], SERPIL PAKSOY[2], SAHVER EGE HISMIOGULLARI[3], MURAT ABDULKADIR KUS[4], FATMA BAHAR SUNAY[5], BURAK GULCEN[1], EMRAH OZCAN[1]

[1]Department of Anatomy, Faculty of Medicine, University of Balikesir, 10145, Cagis Yerleskesi, Balikesir, Turkey - [2]Department of Pathology, Faculty of Medicine, University of Balikesir, 10145, Cagis Yerleskesi, Balikesir, Turkey - [3]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Balikesir, 10145, Cagis Yerleskesi, Balikesir, Turkey - [4]Health of College, University of Mehmet Akif Ersoy, 15000, Burdur, Turkey - [5]Department of Histology and Embryology, Faculty of Medicine, University of Balikesir, 10145, Cagis Yerleskesi, Balikesir, Turkey

ABSTRACT

***Introduction**: An attempt was made to examine the protective effects of omega-3 fatty acids against liver injury induced by carbon tetrachloride (CCl_4) in rats.*

***Materials and methods**: Twenty-one male Wistar rats were divided in three groups. Group I was chosen as control, Group II subcutaneously injected every other day with CCl_4 for 1 month, whereas rats in Group III were received daily ω-3 fatty acids via intragastric gavage while exposed to CCl_4 for 1 month. Finally, all animals were killed by decapitation and blood samples were taken. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total and conjugated bilirubin levels were measured. Additionally, hepatic superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) contents were determined. For histopathological evaluation, the liver samples from experimental and control groups were processed for light microscopy.*

***Results**: All serum biochemical parameters and the hepatic MDA content were significantly higher in animals treated with CCl_4 than in the controls, while SOD and GSH-Px values were significantly decreased in these animals. Moreover, administration of CCl_4 alone caused histopathologically prominent damage in the liver compared to the control group. Daily ω-3 fatty acids treatment significantly reduced serum biochemical parameters and hepatic MDA levels. These fatty acids also reduced serum SOD and GSH-Px activities in rats received CCl_4 plus ω-3 fatty acids. Furthermore, the histopathological changes induced by CCl_4 were attenuated with administration of ω-3 fatty acids.*

***Conclusion**: These findings suggested that ω-3 fatty acids are involved in protecting liver against CCl_4 toxicity.*

***Keywords**: liver, hepatic toxicity, oxidative stress, superoxide dismutase, animal model.*

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Introduction

The molecular mechanism of carbon tetrachloride (CCl_4) induced- hepatotoxicity has been well reported in rats by generation of free radicals[(1)]. The trichloromethyl free radical (CCl_3), which is occurred in the metabolism of CCl_4 by cytochrome P-450 enzyme system then reacting rapidly with molecular oxygen to produce the trichloromethyl peroxyl radical (CCl_3O_2) radical. This toxic radical is responsible for the removal of hydrogen atoms from unsaturated fatty acids of phospholipids present in the cell membrane, causing lipid peroxidation in the hepatocytes and others.

It has been documented that lipid peroxidation was the main mechanism in the pathogenesis of

liver damage induced by CCl_4[2]. Malondialdehyde (MDA), which is a stable metabolite of the free radical associated with lipid peroxidation cascade considered as marker of lipid peroxidation[3]. Furthermore, CCl_4 causes increasing levels of hepatic enzymes, which are markers of hepatocytes injury[2,4]. Beside these, histopathological changes take play in the liver after CCl_4 administration[3,5,6].

Omega-3 essential fatty acids (ω-3 EFA) consist of eicosapentaenoic acid (EPA, C20:5n-3), docosahexaenoic acid (DHA, C22:6n-3) and α-linolenic acid (ALA, C18:3n-3). EPA and DHA are the members of polyunsaturated fatty acids (PUFA) and found predominantly in the fish oil, whereas ALA is present in the vegetal sources such as soybean and linseed oil[7-9]. EPA could be produced from ALA by several enzymatic chain reactions, EPA can be produced from ALA and finally DHA can be produced from EPA, just only 5% of ALA could enter above-mentioned metabolic pathway. EPA level of tissues increases when ALA administration is high on a diet, whereas DHA level is not changed[10].

It has been reported that DHA treatment significantly protects from several pathologies such as glomerulonephritis, rheumatoid arthritis, autoimmune diseases, allergic asthma, hypertension, cardiovascular diseases, and as adjuvant in cancer therapy[11,12]. As well as, it has been shown that ω-3 fatty acids have antioxidant property[9,12-14].

The present study was conducted to examine the protective effects of ω-3 fatty acids against CCl_4-induced hepatotoxic injury in rats at biochemical and histopathological levels.

Materials and methods

Animals

The Animal Ethics Committee of the Firat University approved the experimental protocol and the animals were cared in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals. Twenty one male Wistar-Albino rats, aged five months and weighing 250-300 g were housed in separate cages at 25°C and subjected to a 12:12-h light:dark cycle. The rats were randomly divided into three equal groups: Control group (Group 1), subjected to subcutaneous (sc) 1 ml pure olive oil administration on every other day for 4 weeks; CCl_4 group (Group 2), subjected to sc CCl_4 (EM Science, Cherry Hill, NJ, USA) injection, 0.5 ml/kg of body weight in 1 ml olive oil on every other day for 4 weeks, and CCl_4 + Omega group (Group 3), subjected to sc CCl_4 injection, 0.5 ml/kg in olive oil on every other day for 4 weeks plus omega-3 fatty acids (400 mg/kg, *Marincap capsule®*) given by gavage every day for 4 weeks. The fatty acid composition of Marincap capsule (500 mg) is formed by EPA (18%) and DHA (12%).

All animals were allowed *ad libitum* food consumption of standard laboratory diet and had free access to water throughout the experimental period. The rats in all groups were anesthetized on day 28 of the CCl_4 administration. with sodium pentobarbitone (6mg/100 gr of body weight, intraperitoneal). Blood samples, approximately 3 ml from each animal, were collected from abdominal aorta into routine biochemical tubes for biochemical analyses. Then animals were hepatectomized and the liver was separated for further biochemical analysis and histopathological examination.

Determination of Biochemical Parameters in Serum

The blood samples were collected into tubes, allowed to clot and the serum was removed by centrifugation at 2000 *g* for 10 min. All serum samples were sterile, hemolysis-free, and were kept at 4ºC before determination of the biochemical parameters.

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin and conjugated bilirubin levels were measured with an AU600 multiparameter analyzer (Olympus, Hamburg, Germany).

Biochemical and Histopathological Evaluation of The Liver Tissues

After weighing, the liver samples (1 g) were washed twice with a cold saline solution, placed into glass bottles, labeled and stored at -30 ºC for eventual determination of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) production. The other liver tissue specimens were used for histopathological examination.

Biochemical Analysis of The Liver

For biochemical analysis, the liver samples were homogenized in four volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 0.50 ml/l Triton X-100 with a homogenizer (IKA Ultra-

Turrax T 25 Basic, Germany) for 2 min at 13,000 rpm. All procedures were performed at 4°C. Tissue MDA levels were determined on the homogenate. The tissue homogenates were centrifuged at 5000 *g* for 60 min to remove debris and the clear supernatant fluids were separated and kept at -40°C until the enzyme activity measurements were performed (about a week later).

Determination of Liver Superoxide Dismutase Activity

Total (Cu-Zn and Mn) SOD activity (EC 1.15.1.1) was determined based on the method of Sun et al.[15]. The principle of the method is based on the inhibition of Nitro Blue Tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1 ml of ethanol-chloroform mixture (5:3, v/v) was added into the same volume of sample and centrifuged. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. The SOD activity was expressed as U/g protein.

Determination of Liver Glutathione Peroxidase Activity

Glutathione peroxidase (GSH-Px, EC 1.6.4.2) activity was measured by the method of Paglia and Valentine[16]. The enzyme reaction in the tube containing NADPH, reduced glutathione (GSH), sodium azide and glutathione reductase were initiated by addition of H_2O_2, and the change in absorbance at 340 nm was monitored by a spectrophotometer. The activity was expressed as U/g protein.

Determination of Liver Malondialdehyde Level

The tissue MDA level was determined by using a method of Esterbauer and Cheeseman[17], based on reaction with thiobarbituric acid (TBA) at 90-100°C. In the TBA test reaction, MDA and TBA react to produce a pink pigment with absorption maximum at 532 nm. The reaction was performed at pH 2-3 and 90°C for 15 min. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was centrifuged and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water-bath for 10 min. After cooling, the absorbance was read at 532 nm. The results were expressed as nmol/g wet tissue, by reference to a standard curve prepared from measurements made with a standard solution (1,1,3,3-tetramethoxypropane).

Histopathological Examination of The Liver

The liver tissue specimens were fixed in neutral formalin solution (10%). The tissue specimens were embedded in paraffin wax and sectioned (thickness, 5 μm). For histopathological evaluation, the sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome, and examined with Olympus BX-51 light microscope.

Statistical Analysis

All biochemical data are expressed as mean ± standard deviation (SD). The quantitative data were tested for normal distribution by using the Kolmogorov-Smirnov test. Since all data were found to be normally distributed, within group comparisons were made by using one-way ANOVA followed by an LSD post-hoc test and the level of significance was set at $p<0.05$ in statistical analyses performed with SPSS (Statistical Package for the Social Sciences) 16.0 for Windows Software (SPSS Inc., Chicago, IL, USA)

Results

The means of the serum biochemical parameters of the groups are shown in Table 1.

PARAMETER	Control	CCl_4	CCl_4 + ω-3
AST (U/L)	239.25 ± 22.10	798.35 ± 74.66*	244.25 ± 16.45
ALT (U/L)	56.63 ± 8.11	620.65 ± 68.09*	60.30 ± 4.00
ALP (U/L)	144.22 ± 11.82	754.33 ± 54.20*	181.56 ± 16.24
T. bilirubin (mg/dl)	0.24 ± 0.01	0.61 ± 0.03*	0.29 ± 0.02
C. bilirubin (mg/dl)	0.07 ± 0.01	0.24 ± 0.02*	0.09 ± 0.01

Table 1: Mean values of serum biochemical parameters in the groups.
*Values are expressed as Mean±SD. n=7 per group. *p<0.05 vs. other groups. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP)*

The values of the serum AST, ALT, ALP, total bilirubin and conjugated bilirubin in group treated with CCl_4 were significantly higher than control group ($p<0.05$). Administration of ω-3 fatty acids along with CCl_4 significantly decreased the elevations of serum AST, ALT, ALP, total bilirubin and conjugated bilirubin ($p<0.05$).

SOD, GSH-Px enzyme activities and MDA levels in the liver tissue samples were depicted in Figures 1, 2 and 3. SOD and GSH-Px enzyme values were significantly decreased in CCl_4-treated group, compared with control group ($p<0.05$) (Figures 1 and 2).

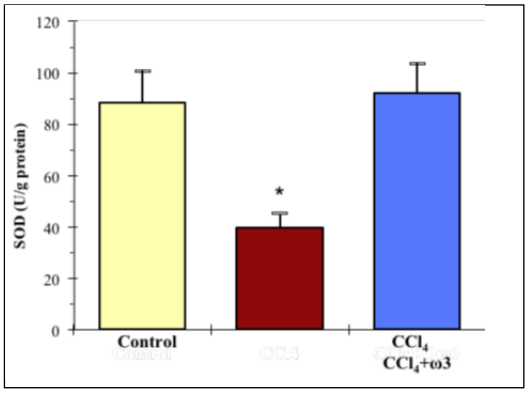

Figure 1: Superoxide dismutase (SOD) activities in the liver tissues of rats divided into control, carbon tetrachloride (CCl_4) and carbon tetrachloride plus omega-3 (CCl_4+ω3) subgroups. *$p<0.05$ significant differences compared to other groups.

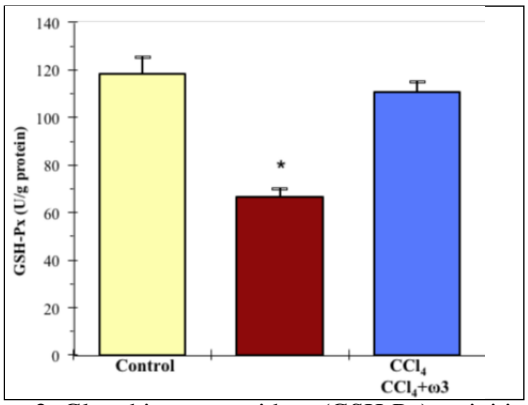

Figure 2: Glutathione peroxidase (GSH-Px) activities in the liver tissues of rats divided into control, carbon tetrachloride (CCl_4) and carbon tetrachloride plus omega-3 (CCl_4+ω3) subgroups. *$p<0.05$ significant differences compared to other groups.

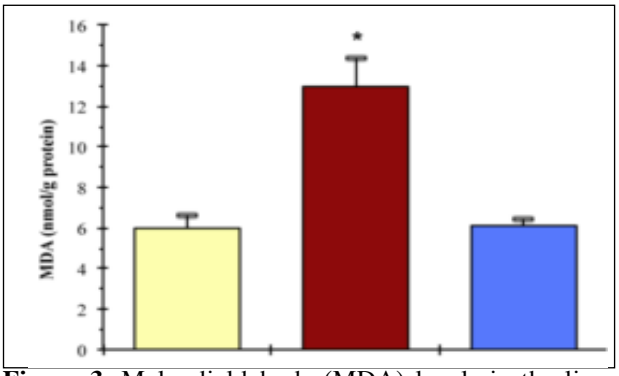

Figure 3: Malondialdehyde (MDA) levels in the liver tissues of rats divided into control, carbon tetrachloride (CCl_4) and carbon tetrachloride plus omega-3 (CCl_4+ω3) subgroups. *$p<0.05$ significant differences compared to other groups.

One of the important oxidative stress parameters is MDA, which is considered as a marker for lipid peroxidation of the tissue. MDA levels in the CCl_4-administered group were significantly higher than control group ($p<0.05$) (Figure 3).

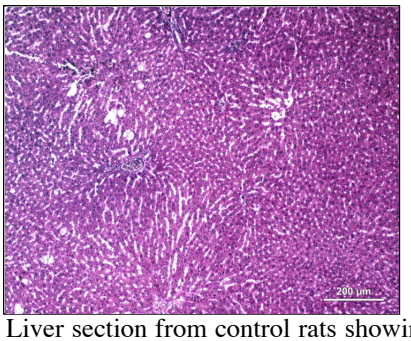

Figure 4: Liver section from control rats showing a normal tissue structure. H&E. Scale bar, 200 µm.

In contrast, administration ω-3 fatty acids along with CCl_4 treated rats led to the increases in SOD and GSH-Px enzyme activities whereas MDA levels were decreased compared with CCl_4-treated rats ($p<0.05$) (Figures 1, 2 and 3).

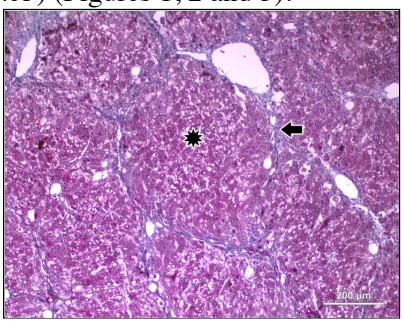

Figure 5: Liver tissue in animals treated with carbon tetrachloride showing a classic cirrhotic appearance with presence of regenerative nodules (asterisk) and massive fibrosis (arrow). Masson's trichrome. Scale bar, 200 µm.

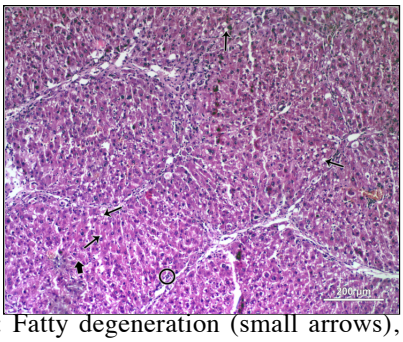

Figure 6: Fatty degeneration (small arrows), mononuclear cell infiltration (circle), microvesicular steatosis and hydropic degeneration (large arrow) were observed in the liver section of carbon tetrachloride -treated rats. H&E. Scale bar, 200 µm.

When the liver tissue structure belonging to animals in control group was examined, it was determined to have an usual microscopic view (Fig. 4). In the tissue specimens of rats exposed to CCl_4 observed pathologic appearances.

Massive fibrosis, fatty degeneration, mononuclear cell infiltration and formation of regenerative nodules were observed in the tissue specimens of rats treated with CCl_4. Additionally, microvesicular steatosis and hydropic degeneration in hepatocytes were seen in this group (Figures 5 and 6).

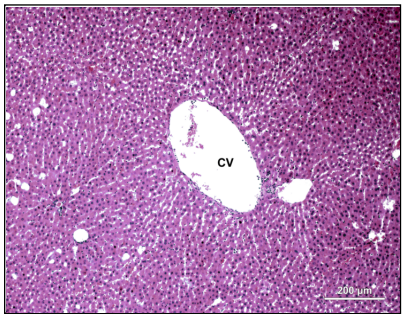

Figure 7: Liver section of rats treated with carbon tetrachloride and omega-3 fatty acids. Except for dilatation of central vein, normal lobular appearance was seen in this group. cv: central vein. H&E. Scale bar, 200 µm.

However, administration of ω-3 fatty acids along with CCl_4 ameliorated the histopathological findings of CCl_4 treated rats. Except for dilatation of central vein, a normal lobular appearance was seen in this group (Fig. 7).

Discussion

The mechanism of hepatotoxicity induced by CCl_4 involves peroxidation of fatty acids present in the hepatocytes membrane which lead to destruction of the cell and their intracellular organelles. CCl_4 is biotransformed by liver cytochrome P450 enzymes to form the trichloromethyl free radical which can cause membrane lipid peroxidation and disturbs Ca^{2+} homeostasis leading to hepatocellular injury[4,18-20]. The administration of CCl_4 to rats cause severe liver injury which was confirmed in our study by histopathological changing accompanied with the increases in activities of serum hepatic enzymes AST, ALT, ALP, total bilirubin and conjugated bilirubin[2,21,22].

One of the most important oxidative stress parameters is the MDA level in the liver. This is in agreement with our results that administration of CCl_4 to rats resulted in a significant increase in the liver MDA contents[23-25]. The antioxidant defense system takes an important role in maintaining biochemical cell function against oxidative stress. The previous experimental studies have shown that antioxidant enzymes such as SOD and GSH-Px protect cells from oxidative stress[18]. The present study established that administration of CCl_4 caused the decreased levels of SOD and GSH-Px enzymes in the livers. These decreases demonstrated that the antioxidant defense mechanisms in the hepatocytes destroyed by administration of CCl_4. This is in agreement with the previous experimental studies[24,25].

The liver injury induced by CCl_4 was also confirmed in our study by light microscope examination. The findings in the present study, the liver of CCl_4 treated rats have shown characteristic morphological changings such as massive fibrosis, fatty degeneration, mononuclear cell infiltration and formation of regenerative nodules. Furthermore, microvesicular steatosis and hydropic degeneration in the hepatocytes of this group were also seen. The previous experimental studies have reported that administration of CCl_4 led to apoptosis, fibrosis, fatty degeneration, mononuclear cell infiltration, steatosis, hemorrhage, hydropic degeneration and formation of regenerative nodules in liver tissue[3,26,27]. In terms of histopathological changes which CCl_4 administration caused in the liver, our findings are in agreement with the previous studies.

In order to carry out cellular functions such as the cell membrane, endoplasmic reticulum, and mitochondrion are necessary to take fatty acids with diet, particularly omega-3. These fatty acids have important biological activities such as contributing structure of cell membrane, having profound effects on biological responses. It has been reported that these lipids have also important physiological affects on cellular movements, membrane stability, membrane fluidity, and receptor morphology as well[28]. In their study, Yilmaz et al.[12] have noted that ω-3 fatty acids have anti-inflammatory, anti-hypertensive and anti-hyperlipidemic effects, therefore, showing the protective effects for the organism.

Another study which was conducted by Clark and Parbtanl[29], omega-3 fatty acids attenuated inflammatory diseases by inducing cytokines, and also protect the organism from renal diseases. Omega-3 fatty acids have also been shown to cause a decreased oxidative damage in various tissues[9,13,14]. In addition, ω-3 fatty acids have also protective effects on the liver tissue in experimental studies[12, 30-32].

The findings in the present study demonstrated that administration of omega-3 fatty acids decreased the progression of liver damage in rats treated with CCl_4 since ω-3 fatty acids significantly attenuated the elevated levels of serum AST, ALT, ALP, total bilirubin and conjugated bilirubin, which are indices of hepatocytes injury. In addition, the rats that were exposed to CCl_4 along with ω-3 fatty acids had increased SOD and GSH-Px enzyme levels and decreased MDA levels when compared to CCl_4-treated group. The protective effects of ω-3 fatty acids confirmed by the biochemical results in CCl_4-induced rat liver, as well as histological investigation. Except for dilatation of central vein, histopathological data also demonstrated that administration of ω-3 fatty acids reduces CCl_4-caused inflammation and maintain a normal lobular appearance.

In conclusion, according to the biochemical and histopathological findings in our study, we have shown that ω-3 fatty acids prevent CCl_4-induced liver injury in rats.

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Corresponding author
Assist. Prof. Dr. OMUR KARACA SAYGILI
Department of Anatomy, Faculty of Medicine, Balikesir University, Cagis Yerleskesi
10145, Balikesir
(Turkey)