

## Effects of cod liver oil on tissue antioxidant pathways in normal and streptozotocin-diabetic rats

Tuğba Hünkar<sup>1</sup>, Fugen Aktan<sup>2</sup>, Aslı Ceylan<sup>1</sup>, Çimen Karasu\*<sup>1</sup>  
and (Antioxidants in Diabetes-Induced Complications) The ADIC Study Group

<sup>1</sup>Department of Pharmacology, Faculty of Pharmacy, Ankara University, Ankara, Turkey

<sup>2</sup>Department of Biochemistry, Faculty of Pharmacy, Ankara University, Turkey

Lipid disorders and increased oxidative stress may exacerbate some complications of diabetes mellitus. Previous studies have implicated the beneficial effects of some antioxidants, omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the protection of cells from the destructive effect of increased lipids and lipid peroxidation products. This study, therefore, was designed to investigate the effects of cod liver oil (CLO, Lysi Ltd. Island), which comprises mainly vitamin A, PUFAs, EPA and DHA. Effects were monitored on plasma lipids, lipid peroxidation products (MDA) and the activities of antioxidant enzymes, glutathione peroxidase (GSHPx) and catalase in heart, liver, kidney and lung of non-diabetic control and streptozotocin (STZ)-induced-diabetic rats. Two days after STZ-injection (55 mg kg<sup>-1</sup> i.p.), non-diabetic control and diabetic rats were divided randomly into two groups as untreated or treated with CLO (0.5 ml kg<sup>-1</sup> rat per day) for 12 weeks. Plasma glucose, triacylglycerol and cholesterol concentrations were significantly elevated in 12-week untreated-diabetic animals; CLO treatment almost completely prevented these abnormalities in triacylglycerol and cholesterol, but hyperglycaemia was partially controlled. CLO also provided better weight gain in diabetic animals. In untreated diabetic rats, MDA markedly increased in aorta, heart and liver but was not significantly changed in kidney and lung. This was accompanied by a significant increase in both GSHPx and catalase enzyme activities in aorta, heart, and liver of diabetic rats. In kidney and lung, diabetes resulted in reduced catalase while GSHPx was significantly activated. In aorta, heart, and liver, diabetes-induced changes in MDA were entirely prevented by CLO treatment. In the tissues of CLO-treated diabetic animals, GSHPx activity paralleled those of control animals. CLO treatment also caused significant improvements in catalase activities in every tissue of diabetic rats, but failed to affect MDA and antioxidant activity in control animals. The current study suggests that the treatment of diabetic rats with CLO provides better control of glucose and lipid metabolism, allows recovery of normal growth rate, prevents oxidative/peroxidative stress and ameliorates endogenous antioxidant enzyme activities in various tissues. Because CLO contains a plethora of beneficial compounds together, its use for the management of diabetes-induced complications may provide important advantages. Copyright © 2002 John Wiley & Sons, Ltd.

KEY WORDS — cod liver oil; streptozotocin-diabetes; aorta; heart; liver; kidney; lung; oxidative stress; catalase; glutathione peroxidase

### INTRODUCTION

Increased production of reactive oxygen species seems to be an important biochemical modification in some pathological events that cause complications

accompanying diabetes such as atherosclerosis, ischaemic heart disease, nephropathy, pulmonary disease, and fatty liver.<sup>1</sup> Hyperglycemia in diabetes mellitus generates free radicals by mechanisms that are thought to involve metal-catalysed oxidation of glucose, oxidative degeneration, and protein glycation.<sup>2</sup> Diabetes also disturbs natural antioxidant defence systems, changing antioxidant enzyme activities in various tissues including aorta,<sup>3,4</sup> heart,<sup>3,5,6</sup> liver,<sup>5-7</sup> lung<sup>8</sup> and kidney.<sup>9</sup> Previous studies have shown that antioxidants, including vitamins C, E, A,<sup>10,11</sup>

\* Correspondence to: Dr Ç. Karasu, Department of Pharmacology, Faculty of Pharmacy, Ankara University, 06100, Tandoğan, Ankara, Turkey. Tel: (90) 312 2126805 Ext. 2226. Fax: (90) 312 2131081. E-mail: karasu@pharmacy.ankara.edu.tr

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coenzyme Q10,<sup>6</sup> probucol,<sup>12</sup> and alpha-lipoic acid,<sup>4</sup> have some capacity in preventing or reversing disturbances in tissue antioxidant defence in diabetic animals.

On the other hand, an increase in circulating lipids may be a reason for their increased peroxidation in diabetes. In this regard, cod liver oil (CLO) is of interest not only because of its role in lipid homeostasis, but also in the treatment of some diseases such as cardiovascular, metabolic and renal disease.<sup>13</sup> CLO is a particularly rich source of omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). As major constituents of membrane lipids, EPA and DHA are vital to the proper functioning of various tissues such as blood vessels, heart, eyes and nerves. They are known to lower lipids, decrease triacylglycerol, and increase HDL in plasma and, have antithrombotic and anti-inflammatory effects, provide protection against arteriosclerosis, prevent life-threatening arrhythmias and facilitate the normal growth, development and function of some tissues.<sup>14-17</sup> PUFAs that have anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic, and vasodilatory properties also, play an important role in human development, physiology and in the treatment and prevention of certain diseases such as coronary heart disease, hypertension, Type-2 diabetes and renal disease.<sup>13,16,18</sup> The results of a previous study indicated that in fish, dietary lipids depress hepatic lipogenic activity as well as lipid peroxidation products by maintaining the endogenous antioxidant capacity.<sup>17,19</sup> PUFAs have been shown to lead to effective changes in vascular risk factors, and to reduce *in vivo* oxidant stress in humans and animals with hypertension.<sup>15,20</sup> Reports also indicated that vitamin A as an antioxidant in the CLO could account for the beneficial effects of CLO. CLO also has a protective effect against Type 1 diabetes.<sup>21</sup> Although a large amount of research has been conducted in this area, the effects of CLO on oxidative stress and endogenous antioxidant status have not previously been examined in experimental diabetes. Thus, the aim of the present study was to assess whether diabetes-induced changes in parameters of glucose and lipid metabolism, oxidative stress and tissue antioxidant defence mechanisms are prevented by CLO.

## MATERIALS AND METHODS

### *Induction of diabetes and the treatment protocols*

Male Wistar rats, body weight 250–300 g were fed a standard rat chow diet and had access to water *ad*

*libitum*. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 55 mg kg<sup>-1</sup> body weight) to animals fasted overnight. Blood samples for biochemical measurements were taken from the tail vein. The rats with blood glucose of 250 mg dl<sup>-1</sup> or more were considered to be diabetic. The experimental groups comprised control and diabetic treated or untreated with cod liver oil (CLO, Lysi Ltd. Island, 0.5 ml kg<sup>-1</sup> day<sup>-1</sup>, orally). The dose regimen of the CLO was chosen according to some previous studies.<sup>22</sup> Animals were treated for a period of 12 weeks beginning 48 h after STZ injection.

### *Blood and tissue analysis*

Blood glucose concentrations and body weights were determined weekly. Plasma triacylglycerol and cholesterol concentrations and tissue (aorta, heart, liver, kidney, lung) analyses were assessed at the end of the treatment period. Blood glucose concentrations were measured by an Ames glucometer (Glucometer III, Bayer Diagnostics, France); plasma triacylglycerol and cholesterol concentrations were measured using a commercially available enzyme kit (Wako, Osaka, Japan).

Malondialdehyde (MDA), an end-product of lipid peroxidation, was measured fluorometrically in tissue homogenates.<sup>23</sup> A volume of 200 µl of tissue homogenate was heated at 95°C for 60 min together with 250 µl 42 mM 2-thiobarbituric acid and 750 µl 0.19 mM H<sub>3</sub>PO<sub>4</sub>. Standard samples were prepared from malondialdehyd-bis-(diethylacetal). The samples were precipitated with a mixture of methanol and 1 M NaOH and centrifuged at 4000 r.p.m. for 5 min. Fluorescence was then measured in the supernatant, the excitation wavelength was 532 nm and emission wavelength was 553 nm.

Catalase was measured spectrophotometrically by the method of Aebi.<sup>24</sup> In a cuvette containing 1.9 ml 50 mM phosphate buffer (pH 7.0), 0.1 ml of tissue supernatant was added. The final volume of the mixture was made up to 2.0 ml by adding more buffer solution. The reaction was started by the addition of 1.0 ml of freshly prepared 30 mM H<sub>2</sub>O<sub>2</sub>. The rate of decomposition of H<sub>2</sub>O<sub>2</sub> was measured spectrophotometrically at 240 nm. The enzyme activity for tissues was expressed as  $k s^{-1} mg^{-1}$  protein, where  $k$  is the first-order rate constant.

The method of Lawrence and Burk was used to measure glutathione peroxidase (GSH-Px) activity.<sup>25</sup> The assay mixture consisted of 2.0 ml of 75 mM phosphate buffer (pH 7.0), 50 µl of 60 mM glutathione, 0.1 ml of 30 units ml<sup>-1</sup> glutathione reductase, 0.1 ml

of 15 mM disodium salt of EDTA, 0.1 ml of 3 mM NADPH and the appropriate amount of tissue supernatant to a final volume of 3.0 ml. The reaction was started by the addition of 0.1 ml of 7.5 mM  $\text{H}_2\text{O}_2$ . The rate of change of absorbance during the conversion of NADPH to  $\text{NADP}^+$  was recorded spectrophotometrically at 340 nm for 3 min. GSH-Px activity for tissues was expressed as  $\mu\text{mol}$  of NADPH oxidized to  $\text{NADP}^+$   $\text{min}^{-1} \text{mg}^{-1}$  protein.

The protein content of homogenates and supernatants was measured by the method of Lowry *et al.*<sup>26</sup>

#### Drugs and statistical analysis

All chemicals except CLO were purchased from Sigma Chemical (St. Louis, MO, USA). CLO was supplied from Cansin Medical, a company in Turkey, which distributes the products of Lysi Co. Island. Data are expressed as mean  $\pm$  SEM. They were first subjected to Bartlett's test for homogeneity of variances and were given a log transformation if necessary. One-way analysis of variance was then performed, followed by the Student–Newman–Keul's test to estimate the significance of differences for individual between-group comparisons.

## RESULTS

The initial body weights were similar in all groups. One week after STZ injection, the body weights of diabetic rats both treated and untreated, were significantly less than non-diabetic normal animals (Figure 1A). Treated or untreated diabetic animals kept a decreased body weight status until their sacrifice. However, CLO treatment exerted beneficial effects on weight gain of diabetic animals, but did not significantly affect the weight gain profile of normal rats during the treatment period (Figure 1A).

Blood glucose concentrations were about three-fold higher in diabetic rats than in control rats (Figure 1B). CLO treatment induced a significant fall in blood glucose levels of diabetic animals. No significant difference was found between blood glucose concentrations in control rats treated with CLO and the

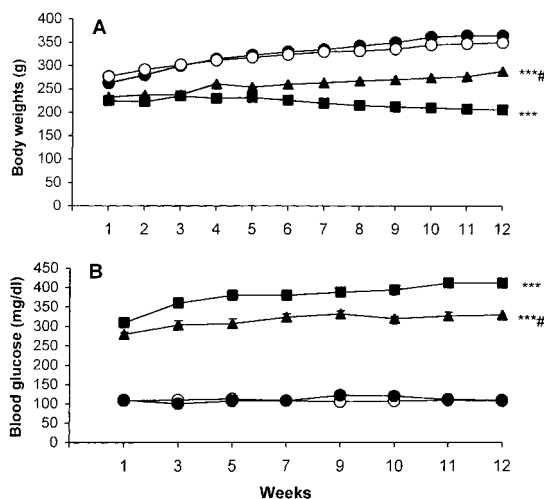


Figure 1. Body weight (A) and blood glucose (B) profiles of untreated control (●), CLO-treated control (○), untreated diabetic (■), CLO-treated diabetic (▲) rats. Data (mean  $\pm$  SEM) were analysed by ANOVA and between groups differences for each variable were tested using Newman–Keul's test. \*\*\* $P < 0.001$  versus control; # $P < 0.001$  versus untreated diabetic rats

corresponding untreated group. Although CLO was found to be effective in decreasing blood glucose levels, at the end of the treatment, diabetic rats were still hyperglycaemic (Figure 1B).

Plasma triacylglycerol and cholesterol concentrations markedly increased in untreated diabetic rats. CLO treatment completely prevented these abnormalities in plasma triacylglycerol and cholesterol concentrations. CLO treatment also decreased plasma triacylglycerol in control animals (Table 1).

Values of antioxidant markers in normal rat tissues are shown in Figure 2. Malondialdehyde (MDA), an end-product of lipid peroxidation, was markedly elevated in aorta, heart and liver of diabetic rats compared with the controls (Figure 2). These increases were completely prevented by CLO. MDA concentrations in kidney and lung were similar in control and diabetic rats treated with or without CLO (Figure 2).

Vascular, cardiac and hepatic catalase and GSHPx activities were found to be significantly increased in

Table 1. Plasma cholesterol and plasma triacylglycerol concentrations of untreated control ( $n = 6$ ), CLO treated-control ( $n = 6$ ), untreated-diabetic ( $n = 8$ ), and CLO-treated diabetic ( $n = 8$ ) rats

	Untreated-control	CLO treated-control	Untreated-diabetic	CLO treated-diabetic
Plasma cholesterol ( $\text{mg dl}^{-1}$ )	$61 \pm 3^{\#}$	$58 \pm 4^{\#}$	$102 \pm 6^{***}$	$68 \pm 5^{\#}$
Plasma triacylglycerol ( $\text{mg dl}^{-1}$ )	$80 \pm 3^{\#}$	$60 \pm 4^{* \#}$	$232 \pm 13^{***}$	$92 \pm 14^{* \#}$

Data are reported as means  $\pm$  SEM.

\* $P < 0.05$ ; \*\*\* $P < 0.001$  vs. untreated-control; # $P < 0.001$  vs. untreated-diabetic.

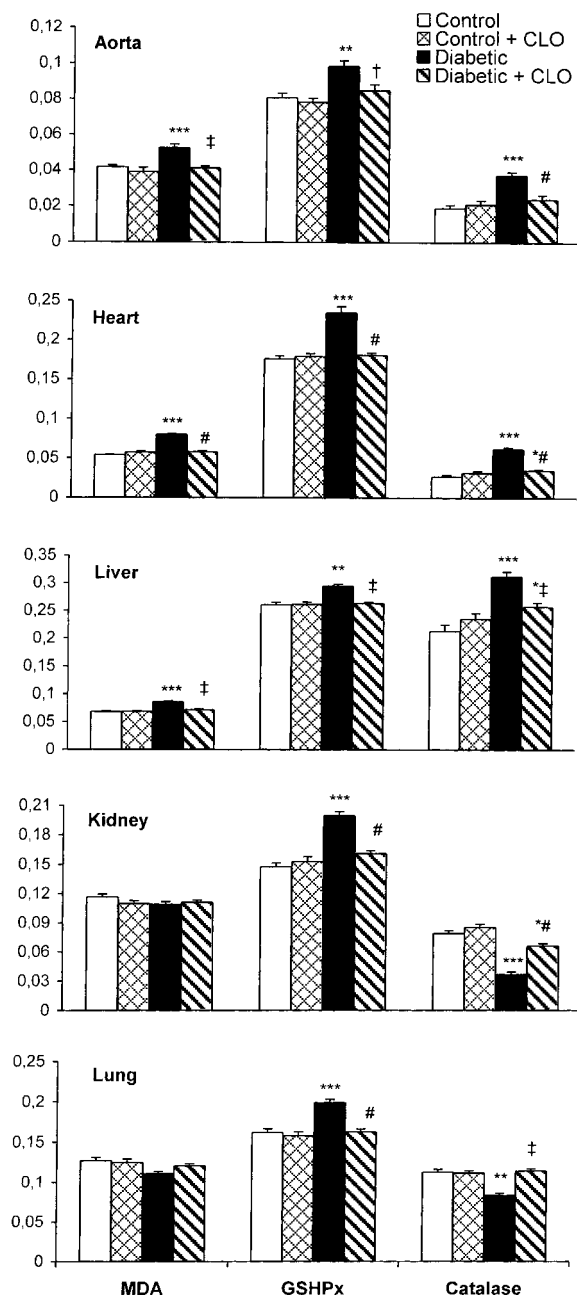


Figure 2. Effects of cod liver oil (CLO) treatment (12 weeks) on an oxidative stress marker and antioxidant enzymes in aorta, heart, liver, kidney and lung of control (normal), diabetic rats. All bars represent means and standard errors of six to eight animals per group. Malondialdehyde, MDA,  $\text{nmol mg}^{-1}$  protein; glutathione peroxidase, GSHPx,  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein; catalase,  $k \text{ s}^{-1} \text{mg}^{-1}$  protein. Data were analysed by ANOVA and between groups differences for each variable were tested using Newman-Keul's test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control; † $P < 0.05$ , ‡ $P < 0.01$ , # $P < 0.001$  versus untreated diabetic rats

untreated diabetic rats compared with control animals (Figure 2). GSHPx activity was also increased in diabetic kidney and lung, while catalase activity was significantly decreased in these diabetic tissues (Figure 2). Treatment with CLO completely or partly normalized almost all of these changes induced by diabetes (Figure 2).

## DISCUSSION

The levels of toxic oxidants, which have been linked to the pathological complications of diabetes,<sup>1</sup> are elevated in diabetic rats due to processes such as glucose oxidation and lipid peroxidation.<sup>2</sup> STZ-induced diabetes in rats, a model of Type-1 diabetes mellitus, is characterized by several irregularities in endogenous antioxidant enzymes.<sup>3-9</sup> In the present study, these aberrations included an increase in MDA and in the activities of GSHPx and catalase in diabetic aorta, heart and liver, whereas in diabetic kidney and lung, these abnormalities were revealed as an increase in GSHPx activity and a decrease in catalase activity with unaltered MDA. Although the results of previous studies regarding the changes in tissue antioxidant levels in the diabetic state are contradictory, the present findings are consistent with those seen by most other researchers.<sup>3-11</sup>

In general, the increase in MDA in the diabetic situation suggests that hyperglycaemia induces the peroxidative reactions in lipids. GSHPx activity was increased in all diabetic tissues in this study, which is supported by our previous findings observed in diabetic aorta and heart.<sup>4,11</sup> This is likely to be an important adaptive response to conditions of increased peroxidative stress since the increased production and the biological availability of  $\text{H}_2\text{O}_2$  has already been established in diabetic tissues.<sup>27</sup> It has been shown that GSHPx is the predominant pathway for degradation of  $\text{H}_2\text{O}_2$  at low concentrations while catalase is operative at higher peroxide concentrations.<sup>28</sup> In this study, in parallel with increased GSHPx, diabetes induced an increase in catalase activity in aorta, heart and liver but not in kidney and lung. The increased catalase activity reflects the increased production of  $\text{H}_2\text{O}_2$ , which agrees with the findings of other observations.<sup>3,27,29</sup> The kidney and lung seem to be partly resistant to peroxidative stress since the MDA contents did not change with the diabetic conditions in spite of decreased catalase and increased GSHPx activities. The mechanism(s) of how MDA was kept at normal levels and why catalase activity was decreased in spite of increased GSHPx in diabetic kidney and lung are not clear.

However the inter-organ variability of changes in tissue catalase may reflect a complex interaction between the diabetes-induced peroxidative stress and the antioxidant systems in various organs.<sup>7</sup> The varying changes in tissue catalase activities may be attributed to the differences in tissue antioxidant capacity and the severity of peroxidative stress among the organs. In this regard, similar tissue-specific changes in the activities of H<sub>2</sub>O<sub>2</sub> and detoxifying enzyme systems have been reported by other investigators in diabetic rats.<sup>7</sup>

We showed that CLO treatment decreases plasma cholesterol, triacylglycerol and lipid peroxidation products, and provides better glucose control in diabetic animals. Although these data are the first evidence obtained with CLO in experimentally-induced Type 1 diabetes, there is some published evidence which shows that the increased levels of plasma triacylglycerol, VLDL, and non-esterified fatty acids in Type 2 diabetic patients are markedly attenuated by treatment with moderate amounts of fish oil. These changes occur without deterioration in blood glucose control and with improved insulin sensitivity.<sup>18</sup> Lipid and glucose lowering effects of EPA, DHA and linolenic acid have also been shown in genetically obese and diabetic KK-Ay mice.<sup>30</sup> The glucose-lowering effect of CLO, observed in this study, parallels previous studies which showed that vitamin D or the omega-3 fatty acids EPA and DHA in the CLO, or both, have a protective effect against Type 1 diabetes,<sup>21</sup> and that the oral supplementation with oils rich in EPA and DHA and omega-6 gamma-linolenic acid and arachidonic acid can protect the animals against chemically-induced diabetes mellitus by enhancing the antioxidant status and suppressing production of cytokines.<sup>31</sup>

Unsaturated fatty acids also have antioxidant properties, and as indicated in our previous study, there may be a relation between its antioxidant properties and the preventive effects on diabetes-induced neuronal dysfunction.<sup>32</sup> The antioxidant properties of unsaturated fatty acids in CLO should be considered as a major component of its beneficial effects on tissue lipid peroxidation and antioxidant enzyme activities observed in the present study. Antihyperlipidaemic properties of CLO may be another important point, which alone can play a major role in the prevention of lipid oxidation. Accordingly, we have previously reported that a lipid-lowering agent, gemfibrozil, significantly reverses the excess lipid peroxidation in plasma and vascular tissue without significant changes in glucose metabolism and antioxidant enzymes in diabetic rats.<sup>33</sup> It is clear that the lowering of circulated lipids provides a reduction in their

amounts as substrate for the oxidation process.<sup>33</sup> In addition, vitamin A, which is a powerful antioxidant component of CLO, may have an important role in the prevention of diabetes-induced deterioration in lipid and glucose metabolism. Recently we showed that vitamin A alone has important benefits in the prevention of hyperglycaemia, providing better control of lipid metabolism, and the maintenance of antioxidant defence in diabetic rats.<sup>11</sup> In this study, the treatment of diabetes with insulin alone was not able to achieve a profound reduction in oxidative, especially peroxidative stress, but the antioxidant vitamin A alone was able to do so.<sup>11</sup> Vitamin A is a member of the lipid-soluble retinoid compounds, plays a central role in the maintenance of many essential biological processes, and shows a function as lipoperoxyl-radical scavenger.<sup>34</sup>

The present results obtained from CLO treatment are in agreement with previous observations with unsaturated fatty acids,<sup>15,17,19,20</sup> lipid-lowering agents,<sup>33</sup> vitamin A,<sup>11</sup> and other antioxidant compounds.<sup>4-6,10</sup> It has been established that insulin treatment, although promoting recovery of normal growth rate and reversing most diabetes-induced biochemical abnormalities, is not able to completely inhibit the glycation of proteins and the formation of advanced glycosylation end-products in diabetic rats,<sup>7,11,5,6</sup> which can increase tissue oxidative stress. Therefore, chronic antioxidant and antihyperlipidaemic therapies as adjuncts to insulin treatment may reduce the risk of diabetic complications. In addition, there is also evidence that antioxidants may act synergistically and that combinations of compounds may be dramatically more effective than any one compound alone.<sup>32</sup> In this respect, CLO, a natural compound, which mainly contains unsaturated fatty acids, EPA, DHA and an antioxidant vitamin may have considerable advantages in its use for the prevention of diabetic complications and in the management of diabetes mellitus.

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