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Cod liver oil in chemically-induced diabetes mellitus in rats

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Alloxan induces diabetes in experimental animals through the selective damage of pancreatic β-cells. Cod liver oil (CLO) is an important source of long-chain ω-3 fatty acids (eicosapentaenoic and docosahexaenoic acids) and vitamins A, E, and D. In the present study, the possible protective effect of CLO against alloxan-inducing diabetes was investigated in rats. Sixty male albino rats were divided into six groups (ten rats each) as following: Group I (control group), rats fed on a standard diet; Group II (diabetic group), rats injected intraperitoneally with alloxan (75 mg kg^{-1} day^{-1}) for five consecutive days; Group III (CLO group), rats received orally 100 μl of CLO for five consecutive days; Group IV (treated group), rats injected with alloxan for five consecutive days followed by CLO administration for five consecutive days; Group V (protected group), rats received CLO for five consecutive days followed by alloxan injection for five consecutive days, and Group VI (simultaneous group), rats received CLO and alloxan at the same time for five consecutive days. After 30 days from starting the injection, plasma glucose, insulin, tumor necrosis factor-α (TNF-α), interleukine-6 (IL-6), and nitric oxide (NO) were investigated. Results showed that plasma levels of glucose, TNF-α, and IL-6 were significantly elevated, while levels of plasma NO and insulin were significantly decreased in diabetic rats when compared with the control group. Oral administration of CLO (protected and simultaneous groups) ameliorated the deleterious effects of alloxan by lowering glucose, TNF-α, IL-6 and by slightly elevating plasma insulin and NO levels. It is concluded that CLO might prevent alloxan action by suppressing the release of inflammatory cytokines (TNF-α and IL-6) that are involved in β-cell damage and development of diabetes.

Key words: cod liver oil, alloxan, inflammatory cytokines, rat.

INTRODUCTION

Alloxan (2, 4, 5, 6-tetraoxypyrimidine; 5, 6-dioxyuracil) has been commonly utilized as an animal model of insulin-dependent diabetes mellitus (IDDM). Alloxan exerts its diabetogenic action when administered intravenously, intraperitoneally or subcutaneously. The action of alloxan in the pancreas is preceded by its rapid uptake by the insulin-secreting cells (β-cells) (Heikkila *et al*., 1976). Type 1 diabetes is among the most prevalent chronic diseases with onset mostly in childhood. It results from an immune-mediated destruction of the pancreatic β-cells and is linked to genes in the HLA complex on chromosome 6p21 (Atkinson & Maclaren, 1994). However, genetic susceptibility is not sufficient for the development of the disease. Although the environmental triggers of the disease are essentially unknown, early diet is among the strongest candidates, together with viral infections (Akerblom *et al*., 2002). Also, alloxan-induced diabetes is due to a cellular-mediated autoimmune destruction of the β-cells of the pancreas (Atkinson & Maclaren, 1994).

Fish oils are sources rich in docosahexaenoic acid (DHA, C22:6 ω-3) and eicosapentaenoic acid (EPA, C20:5 ω-3) (Johansson *et al*., 1998; Storlien *et al*., 2000). EPA, DHA, and α-linolenic acid (ALA) are

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known as omega-3 polyunsaturated fatty acids (ω-3 PUFA). It is known that DHA taken into the body is mostly delivered to the liver through plasma lipoprotein. Previous studies have shown that DHA oil has been used for the treatment of several pathologies such as glomerulonephritis, rheumatoid arthritis, autoimmune diseases, allergic asthma, hypertension, cardiovascular diseases, and as adjuvant in cancer therapy such as mammary and colon tumors (Ramesh & Das, 1996). Long-chain ω-3 fatty acids become incorporated into the cell membranes and have anti-inflammatory properties that may be relevant for the prevention of type 1 diabetes, such as decreased expression of HLA class II molecules on activated human monocytes (Hughes & Pinder, 2000).

The present study was designed to investigate the protective or treated effect of cod liver oil (CLO) supplementation in alloxan-induced diabetes mellitus in rats.

MATERIALS AND METHODS

Animals

Male Wister albino rats weighing 200 ± 5 g, four to five weeks old were used in this study. The animals were purchased from the Egyptian Organization for Biological and Vaccine Production and kept under normal laboratory conditions. They were fed the standard chow diet *ad libitum*. Cod liver oil (CLO) was purchased from a local pharmacy. Each 1 ml contained 27 mg EPA, 21 mg DHA, 2.1 mg vitamin A, 20 μg vitamin D, and 15.5 mg vitamin E.

The experimental animals were divided into six groups (ten rats each) as following:

Group I (**control group**): The animals were injected intraperitoneally (i.p.) for five consecutive days with 100 μl saline day^{-1}.

Group II (**diabetic group**): The animals were injected daily with alloxan (75 mg kg^{-1} day^{-1}) i.p. for five consecutive days. Blood sugar was estimated every three to four days after the last dose of alloxan to confirm the development of diabetes mellitus (DM). These animals developed DM in approximately two to three weeks after the first injection of alloxan (mean ± SD of fasting blood sugar baseline was 295 ± 15 mg dl^{-1}).

Group III (**CLO group**): The animals received orally 100 μl of CLO for five consecutive days.

Group IV (**CLO-treated diabetic group**): The animals were injected i.p. with alloxan (75 mg kg^{-1} day^{-1}) for five consecutive days. After 21 days, the animals received orally 100 μl CLO for five consecutive days.

Group V (**protected group**): The animals received orally 100 μl of CLO for five consecutive days. This was followed by i.p. injection of alloxan for five consecutive days.

Group VI (**simultaneous-treated group**): The animals received orally 100 μl of CLO and were i.p. injected with alloxan for five consecutive days.

The dose of alloxan for induction of diabetes and the dose of CLO were given according to Suresh & Das (2003). At the end of the experiment (30 days), the animals were weighted and sacrificed under anaesthesia with diethyl-ether. A blood sample was collected and divided into two parts; one part was kept in sodium fluoride for separation of the plasma, and the other part was used for separation of the serum. The plasma glucose levels were determined after sampling. The remainders were kept at –20°C.

Methods

Plasma glucose, plasma lactate, serum total cholesterol (TC), high density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c), and triacylglycerol were estimated by using commercially available kits obtained from Boehringer-Mannheim. The level of plasma insulin was estimated using the ELISA kit (Mercodia Ultrasensitive Mouse Insulin, Sweden). Serum tumor necrosis factor alpha (TNF-α), and interleukine-6 (IL-6) were determined with the ELISA technique using the Diamed microtitre strips diagnostic kit according to Myśliwska *et al*. (1998 a, b). Serum nitric oxide (NO) levels were determined according to the method of Vodovotz (1996).

Statistical analysis

Table data are presented as mean values with standard deviation and figure data as mean values with standard error for each group. Statistical evaluations of the differences between the group mean values were tested by one-way analysis of variance (ANOVA).

RESULTS

Results in Table 1 reveal that there is a significant decrease in body weight of the diabetic rats either treated or not treated with CLO when compared with the control group ($p < 0.01$). The animals that received CLO with alloxan (both protected and simul-

TABLE 1. Body weight, plasma glucose, insulin, and lactate levels in all studied groups (mean ± SD)

Groups	Body weight (g)	Plasma glucose (mg dl^{-1})	Plasma lactate (mg dl^{-1})	Plasma insulin (ng ml^{-1})
Gp I: control	186.8 ± 15.0	86.0 ± 4.9	32.8 ± 4.1	6.5 ± 1.1
Gp II: diabetic	146.5 ± 11.3[a]	295.0 ± 15.6[a]	58.0 ± 6.1[a]	2.1 ± 0.3[a]
Gp III: CLO-group	190.0 ± 17.0	92.0 ± 6.1	35.6 ± 2.1	5.7 ± 1.2
Gp IV: diabetic treated with CLO	156.0 ± 15.0[a]	205.0 ± 11.3[a,b]	46.3 ± 4.7[a,b]	3.1 ± 0.9[a]
Gp V: protected group	160.0 ± 10.0[b]	168.0 ± 5.6[a,b]	41.8 ± 5.1[b]	4.9 ± 1.1[b]
Gp VI: simultaneous	162.0 ± 17.0[b]	180.0 ± 4.2[a,b]	65.0 ± 7.1[a,b]	5.1 ± 1.3[b]

CLO: cod liver oil
a: significant difference from control group
b: significant difference form diabetic group
a, b: significant at $p < 0.05$

taneous treatments) showed significantly less loss of body weight when compared with rats receiving alloxan only.

All animals that received alloxan developed diabetes as shown by the high plasma glucose levels (295 mg dl^{-1}) compared with normal rats (86 mg dl^{-1}). Diabetic rats treated with CLO showed a slight decrease in serum glucose compared with diabetic rats ($p < 0.01$), whereas both protected and simultaneous groups showed a higher decrease than the diabetic group ($p < 0.001$) (Table 1 and Fig. 1A). Animals injected with alloxan, either treated or not treated with CLO, showed a significant increase in the plasma lactate level when compared with control animals ($p < 0.001$). A significant decrease in the plasma lactate levels was observed in the protected and treated

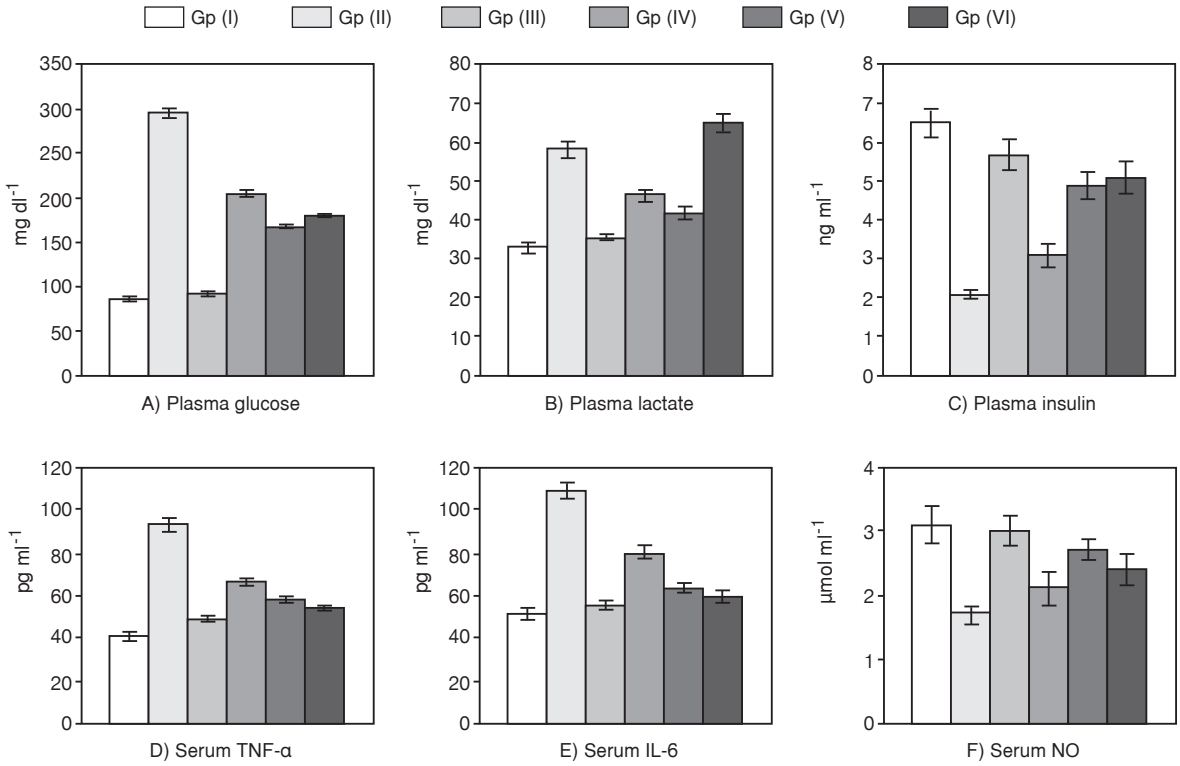

FIG. 1. Plasma glucose, lactate, insulin, and serum TNF-α, IL-6 and nitric oxide levels in all studied groups (mean ± SE). Gp I: control, Gp II: diabetic, Gp III: CLO-group, Gp IV: diabetic treated with CLO, Gp V: protected group, Gp VI: simultaneous.

simultaneously with CLO and alloxan groups, compared with the diabetic group ($p < 0.01$) (Table 1 and Fig. 1B). Plasma insulin levels were significantly decreased in the diabetic rats either treated or not treated with CLO, as compared with normal rats ($p < 0.001$). Oral administration of CLO (either protected or simultaneous with alloxan) showed a slight elevation of the insulin level, as compared with diabetic rats (Table 1 and Fig. 1C).

Table 2 and Fig. 1D and E show that in diabetic rats either treated or not treated with CLO, there was a significant elevation in the levels of serum inflammatory cytokines (TNF-α and IL-6), compared with the control group. The serum levels of TNF-α and IL-6 in the diabetic rats treated with CLO decreased, but they were still much higher than those of the control. Oral administration of CLO in the diabetic rats (treated, protected and simultaneous with alloxan) significantly inhibited the elevation of the TNF-α and IL-6 levels compared with the diabetic group. In contrast, the serum nitric oxide (NO) levels were significantly lower in the alloxan group either treated or not treated with CLO ($p < 0.01$ and $p < 0.001$, respectively) in comparison with the control group. Oral administration of CLO (treated, protected and simultaneous treatment with alloxan) elevated the NO levels as compared with the diabetic group ($p < 0.001$) (Table 2, Fig. 1F).

The lipid profile results showed that diabetic rats either treated or not treated with CLO exhibited a significant elevation in the serum TC, LDL-c ($p < 0.01$) and a significant decrease in the level of HDL-c when compared with the control rats. Oral administration of CLO either protected or simultaneous with alloxan decreased TC and LDL-c, whereas HDL-c was significantly elevated compared with the diabetic rats. Triacylglycerols showed non- significant changes in all studied groups compared with the control rats.

TABLE 2. Serum tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and nitric oxide (NO) levels in all studied groups (mean ± SD)

Groups	TNF-α (pg ml^{-1})	IL-6 (pg ml^{-1})	NO (umol ml^{-1})
Gp I: control	41.0 ± 7.1	52.0 ± 8.4	3.1 ± 0.9
Gp II: diabetic	93.0 ± 9.3[a]	110.0 ± 12.1[a]	1.7 ± 0.4[a]
Gp III: CLO-group	49.0 ± 4.5	56.0 ± 6.5	3.0 ± 0.7
Gp IV: diabetic treated with CLO	66.0 ± 4.5[a]	81.0 ± 8.7[a]	2.1 ± 0.8[a]
Gp V: protected group	58.0 ± 4.1[a,b]	64.0 ± 7.3[a,b]	2.7 ± 0.5[b]
Gp VI: simultaneous	54.0 ± 3.9[a,b]	60.0 ± 8.1[b]	2.4 ± 0.8[b]

CLO: cod liver oil
a: significant difference from control group
b: significant difference form diabetic group
a, b: significant at $p < 0.05$

TABLE 3. Serum total cholesterol (TC), high density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c) and triacylglycerol levels in all studied groups (mean ± SD)

Groups	TC (mg dl^{-1})	HDL-c (mg dl^{-1})	LDL-c (mg dl^{-1})	Triacylglycerol (mg dl^{-1})
Gp I: control	183.0 ± 11.3	48.0 ± 4.1	105.0 ± 7.6	131.0 ± 9.3
Gp II: diabetic	210.0 ± 15.1[a]	31.0 ± 2.9[a]	126.0 ± 6.5[a]	145.0 ± 5.7
Gp III: CLO-group	173.0 ± 11.3	41.0 ± 4.0	110.0 ± 10.1	136.0 ± 2.3
Gp IV: diabetic treated with CLO	215.0 ± 13.3[a]	34.0 ± 3.1[a]	131.0 ± 2.5[a]	149.0 ± 4.6
Gp V: protected group	193.0 ± 11.1[a,b]	39.0 ± 3.2[b]	118.0 ± 8.1[b]	142.0 ± 6.3
Gp VI: simultaneous	181.0 ± 9.5[b]	44.0 ± 3.7[b]	117.0 ± 9.3[b]	135.0 ± 5.5

CLO: cod liver oil
a: significant difference from control group
b: significant difference form diabetic group
a, b: significant at $p < 0.05$

CLO administration in diabetic rats was less effective than that in either the protected or the simultaneous groups. Normal rats orally administrated with CLO for five consecutive days exhibited non-significant changes in all studied parameters when compared with the control group.

DISCUSSION

Alloxan is rapidly taken up by the β-cells and although it is not toxic by itself, the metabolites of alloxan are toxic to the β-islet cells of pancreas (Tomita *et al*., 1974). Alloxan inhibits glucose-stimulated insulin release (Borg *et al*., 1979) and the generation of glucose-derived energy by inhibiting glycolytic flux and pyruvate oxidation (Ishibashi *et al*., 1979). Superoxide radicals generated during the redox cycling of alloxan resulted in the formation of H_2O_2 which forms $OH^{\cdot}$ radicals toxic to the pancreatic β-cells (Heikkila *et al*., 1976). Stene *et al*. (2003) found a significant association between the use of CLO during the first year of life and a lower risk of type 1 diabetes, suggesting that CLO may reduce the risk of type 1 diabetes, perhaps through the effects of long-chain ω-3 fatty acids. CLO or individual fatty acids such as DHA may be candidates for preventive intervention trials.

The present study was designed to evaluate certain inflammatory cytokines during induction of diabetes in rats and the role of CLO in ameliorating the deleterious effect of the diabetogenic substance. Alloxan (75 mg kg^{-1} day^{-1}) was found to cause a significant reduction in the body weight of rats when compared with the control. Animals receiving CLO with alloxan (pre-treatment and simultaneous) increased their body weight compared with the diabetic rats. Suresh & Das (2003) reported that animals which developed alloxan-induced DM showed a significant decrease in body weight due to uncontrolled DM, and animals that received EPA and DHA with alloxan showed a non-significant decrease in weight in comparison with the diabetic rats. Mohan & Das (2001) reported that animals which received alloxan developed type 1 DM approximately 17 days after alloxan injection, as demonstrated by high blood glucose level (350-400 mg dl^{-1}) and low plasma insulin levels compared with the control. This is in agreement with our results that revealed a highly significant elevation in the plasma glucose and a significant decrease in the plasma insulin in diabetic rats as compared with the control. Oral administration of CLO (protected or simultaneous with alloxan) lowered the plasma glucose and elevated the plasma insulin compared with the diabetic group. In diabetic rats, CLO slightly improved the deleterious effects of alloxan. Suresh & Das (2003) reported that, simultaneous and pre-treatment with ALA of rats resulted in 70% and 10% incidence of DM, respectively. Administration of EPA and DHA to the animals also resulted in 70% and 30% of incidence of DM, respectively.

The plasma lactate levels were estimated as a biochemical marker of anaerobic metabolism that occurs in DM (Suresh & Das, 2003). Our results showed a significant increase in the plasma lactate levels in the diabetic rats compared with the control ones. Oral administration of CLO (protected or simultaneous with alloxan) restored the lactate levels. This is in accordance with the results of Suresh & Das (2003), who reported that, EPA and DHA decreased the plasma lactate levels in the alloxan-treated rats.

The prevention of chemically-induced diabetes mellitus in experimental animals by polyunsaturated fatty acids was studied by Mohan & Das (2001). They observed that oral supplementation with oils rich in ω-3 (EPA, DHA) and ω-6 (linolenic, arachidonic) fatty acids could protect the animals against alloxan-induced DM. These oils not only significantly attenuated the chemically-induced DM, but also restored the antioxidant status to a normal range by suppressing production of cytokines. Mohan & Das (1998) found that NO reduces the severity of alloxan-induced DM. Nitric oxide quenches the superoxide anion (Das, 2001), whereas SOD inactivates the superoxide anion. The decreased NO and SOD levels in alloxan-induced DM animals increased the half-life of the superoxide anion. This in turn may contribute to the cytotoxic action of alloxan (Gandy *et al*., 1982).

Table 2 shows that in diabetic rats (either treated or not treated with CLO), there was a significant elevation of the levels of the serum inflammatory cytokines (TNF-α and IL-6) and a significant decrease of the NO levels compared with the control group. Oral administration of CLO in diabetic rats (treated, protected and simultaneous with alloxan) significantly inhibited the elevation of TNF-α and IL-6 and decreased the NO levels.

TNF-α, IL-1 and IL-6 produced by infiltrating macrophages, lymphocytes, and monocytes, damaged the pancreatic β-cells and produced type 1 DM by enhancing the formation of oxygen free radicals, lipid peroxides and aldehydes (Rabinovitch *et al*., 1996a).

Prostaglandin E_2, derived from arachidonic acid, suppresses TNF-α and IL-6 production and is an immunosuppressor (Das, 1981). This suggests that inhibition of TNF-α and IL-6 and enhancement of the production of prostaglandin E_2 may limit the process of pancreatic β-cell damage, and may inhibit the development of type 1 DM in experimental animals (Mandrup-Poulsen *et al*., 1985; Rabinovitch *et al*., 1996b). This study revealed that CLO in the pre-treated and simultaneous groups decreased TC, LDL-c and increased HDL-c when compared with diabetic treated or not treated with CLO. In addition, non-significant changes were detected in the level of serum triacylglycerols in all studied groups when compared with the control group.

Intake of ω-3 fatty acids, either as fish oil or ethyl-ester formulations (selectively enriched in EPA and DHA), is associated with a variety of biochemical changes that might be beneficial for diabetes: reduced triglyceridemia mainly through enhanced triacylglycerol lipolysis, enhanced fatty acid oxidation and raised HDL-c levels; a trend for a more beneficial profile of the LDL particles (Sirtori & Galli, 2002).

Fatty acids (ω-3), possibly acting as "fraudulent fatty acids" can, in fact, activate peripheral fatty acid oxidation (Annuzzi *et al*., 1991). Omega-3 fatty acids are indeed potent activators of peroxisomal proliferator-activated receptors and these may be responsible for enhanced fatty acid utilization. A general consequence is not only reduced triglyceridemia, but also lower insulin resistance and, potentially, improved diabetes control (Fashing *et al*., 1991).

A number of clinical, experimental and epidemiological studies have generally confirmed that intake of ω-3 fatty acid exerts beneficial effects on atherosclerosis development and progression (Hooper *et al*., 2001). These protective effects are attributed to several favorable modifications: changes in plasma lipids, reduction of triglycerides (Harris, 1997), inhibition of the production of arachidonic acid-derived eicosanoids, namely the prothrombotic thromboxane A_2 by activated platelets, and the pro-inflammatory leukotrienes B_2 and C_4 by activated leukocytes (Fischer *et al*., 1986). Dietary fish oils rich in ω-3 fatty acids have been proved to be effective in the lowering of the plasma triacylglycerol and lipoprotein (specially VLDL) levels in experimental animals, thereby being attributed a role in the prevention of cardiovascular diseases (Harris, 1989; Nestel, 1990). Fish or fish oil incorporated into a diet providing 40% of energy as fat, increased total cholesterol, HDL, HDL_2, and LDL, but decreased triacylglycerols (Mori *et al*., 1994). Mori *et al*. (2000) reported that EPA and DHA decreased triacylglycerols and increased fasting insulin. Only DHA increased HDL-c, particularly HDL_2 sub-fraction.

It is concluded that CLO might reduce the risk of type 1 diabetes, perhaps through the effects of long chain ω-3 fatty acids (EPA and DHA). Pre-treatment with CLO showed a better protective action against alloxan-induced DM than simultaneous group did. In addition, diabetic rats treated with CLO did not prevent the deleterious action of alloxan, but may improve the biochemical parameters through a modulatory effect on the inflammatory cytokines.

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