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Evaluation of the effect of n-3 PUFA-rich dietary fish oils on lipid profile and membrane fluidity in alloxan-induced diabetic mice (*Mus musculus*)

Shantal Ganapati Kamat[1] · Ramaballav Roy[1]

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Abstract Marine fishes are important to health due to their high content of polyunsaturated fatty acids particularly those of the omega-3 family. These fatty acids play an important role in various physiological processes and as a consequence they may modulate and even prevent some human diseases. The aim of the present study was to investigate and compare the effect of fish oils of different origins (*Sardinella longiceps*, *Rastrelliger kanagurta* and *Clarias batrachus*) on lipid metabolism and membrane fluidity in diabetes. Alloxan was injected in repetitive doses for 1 month (100 mg/kg body weight every 5th day) to induce diabetes in Swiss albino mice. 10 % *S. longiceps*, *R. kanagurta* or *C. batrachus* fish oil was freshly blended with pellet feed which was provided to diabetic mice for 1 month. The serum lipid profile (serum total cholesterol, triglyceride, HDL, VLDL and LDL) along with liver, kidney and heart tissue lipid profile (i.e. triglyceride, total cholesterol, glycolipid and phospholipid) was analysed. Besides, the enzymatic activity of HMG-CoA reductase, HMG-CoA synthase and glucose-6-phosphate-dehydrogenase along with the membrane fluidity of these tissues was evaluated. Altered tissue lipid composition, enzyme activities and membrane fluidity due to diabetes were returned towards normal with the supplementation of 10 % fish oils. Fish oil from *S. longiceps* brought maximum changes in level of neutral lipid composition in heart, and increased the concentration of phospholipid and decreased the activity of HMG-CoA reductase in comparison with the fish oil from *R. kanagurta* and *C. batrachus*.

✉ Ramaballav Roy
rroy@unigoa.ac.in

[1] Department of Zoology, Goa University, Taleigao Plateau, Goa 403206, India

Keywords Diabetes · Fish oil · Lipid · Membrane fluidity · PUFA · Insulin

Abbreviations

HDL-C	High-density lipoprotein cholesterol
VLDL-C	Very low-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
TG	Triglyceride
MG	Mono-glyceride
DG	Di-glyceride
FFA	Free fatty acid
PL	Phospholipid
TC	Total cholesterol
CE	Cholesteryl esters
HMG-CoA reductase	3-Hydroxy-3-methyl-glutaryl-CoA reductase
HMG-CoA synthase	3-Hydroxy-3-methyl-glutaryl-CoA synthase
G6PD	Glucose-6-phosphate-dehydrogenase
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
PUFA	Polyunsaturated fatty acid
DM	Diabetes mellitus

Introduction

Diabetes mellitus (DM) is possibly the world's fastest growing metabolic disorder and one of the major causes of illness and death [1]. Due to an abnormality in the presence or action of insulin in diabetes, blood glucose level increases and this leads to advanced glycosylated end product formation, to altered cellular lipid metabolism and

to modified membrane lipid packing which can influence the fluidity of the membrane [2]. As insulin plays a central role in the regulation of lipid metabolism, in poorly controlled type 1-diabetes, ketoacidosis, hypertriglyceridemia and reduced HDL concentrations commonly occur [3]. The patient with type 1-diabetes displays four key features of dyslipidemia, i.e. hypertriglyceridemia, a high proportion of small dense low-density lipoprotein cholesterol (LDL), low high-density lipoprotein cholesterol (HDL-C) concentrations and exaggerated postprandial lipemia [4, 5]. The dyslipidaemia associated with diabetes is likely a major risk factor for atherosclerosis and coronary heart disease [6] and is likely to be one of the reasons for the accelerated macro-vascular disease seen in diabetic patients [3].

Fatty acids are an important component of blood, cell and tissue lipids and serve both structural and metabolic roles. Some fatty acids are essential because they cannot be synthesized in the body due to the absence of requisite desaturase enzymes. The two essential fatty acids in humans are alpha-linolenic acid (an omega-3 fatty acid) and linoleic acid (an omega-6 fatty acid) [7–9]. Also the omega-3 and omega-6 families cannot be interconverted in animals. The longer chain, more unsaturated omega-3 PUFA appears to be the most biologically active, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These are found in marine fish and in fish oil supplements [10]. Marine omega-3 PUFAs have been found to have beneficial effects against several diseases including cardiovascular disease, mental illness, obesity, inflammatory diseases and cancer [9]. However, the effects are unclear in diabetes [11, 12] and there is no clear conclusion about the net benefits of administering omega-3 PUFA to diabetic patients. We wished to explore the possible benefits of omega-3 PUFAs in diabetes further and hypothesized that fish oil rich in omega-3 PUFAs would improve lipid metabolism in an animal model of diabetes. We therefore designed research to compare the effects of three laboratory extracted fish oils, from *Sardinella longiceps, Clarias batrachus* and *Rastrelliger kanagurta*, respectively, on lipid metabolism and membrane fluidity in chemically induced diabetes in mice (*Mus musculus*).

Materials and methods

Protocol

Male Swiss albino mice weighing 25 ± 0.5 g were used for the present work and they were maintained as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India). Approval for the work was obtained from Institutional Animal Ethics Committee, Goa University. The animals were fed with a pellet diet containing nearly 3–4 % of lipid *ad libitum*. The animals were divided into 5 groups. Group one (control; NC) mice were injected with 20 µl of saline and the mice in the other four groups were injected with alloxan (100 mg/kg BW in 20 µl saline) to induce diabetes and the diabetic conditions were maintained by repetitive dosage of alloxan every 5th day. Group two (ND) mice were fed with the pellet diet, while mice in the other groups (SD, RD CD) were fed with the same pellet diet but freshly blended with 10 % *Sardinella longiceps* (SD), *Rastrelliger kanagurta* (RD) or *Clarias batrachus* (CD) oils, respectively, for a period of 1 month. After completion of 1 month of dietary treatment, the mice were fasted overnight and then were sacrificed by cervical dislocation to collect blood, liver, kidney and heart tissues. The serum lipids, insulin, free glucose and glycosylated haemoglobin levels were examined. The lipid profile, enzyme activity and membrane fluidity of the tissues were analysed.

Serum lipid profiling

Serum lipids were determined using diagnostic kits manufactured by Crest Biosystem (Coral clinical system Goa, India) and following the method of Trinder. Serum triglyceride, total cholesterol and HDL cholesterol (HDL-C) were assayed and from the values thus obtained the concentrations of VLDL and LDL-C were calculated as follows: VLDL = TG/5 and LDL-C = [Cholesterol-(HDL-C + VLDL)].

Tissue lipid extraction and lipid estimation

Lipid was extracted from tissues (liver, kidney, heart) following the method of Folch et al. [13]. The free fatty acids were analysed using copper reagent [14], triglycerides using chromotropic acid reagent, phospholipids using alcoholic magnesium nitrite [15], total cholesterol using ferric chloride [15] and glycolipids using anthrone reagent [16].

Chromatographic Separations and estimations

Neutral lipids, glycolipids and phospholipids were separated from the total lipid by activated silica gel column chromatography followed by thin-layer chromatography using different solvents/solvent mixture and viewed in an iodine chamber [15]. Each of the spots/fractions was scraped from the plate and analytically quantified. All acylglycerides, namely triglycerides, diglycerides and monoglycerides, were estimated by chromotropic acid reagent [15]. Free cholesterol and esterified cholesterol

were estimated by ferric chloride reagent [15]. Free fatty acids were assayed by sodium dithiocarbamate reagent [14]. All glycolipid and phospholipid spots were estimated by anthrone reagent [16] and ammonium molybdate reagent [15], respectively.

Fatty acid profiling

Total lipid was subjected to saponification by adding 0.7 mL of 10 N KOH in water and 5.3 mL of methanol. The tube was incubated in a 55 °C water bath for 1.5 h with vigorous stirring for 5 s every 20 min to properly permeate, dissolve and hydrolyse the sample. After cooling below room temperature in a cold tap water bath, 0.58 mL of 24 N H_2SO_4 in water was added. The tube was mixed by inversion and with precipitated K_2SO_4 present was incubated again in a 55 °C water bath for 1.5 h with stirring for 5 s every 20 min. After FAME synthesis, the tube was cooled in a cold tap water bath. Three millilitres of hexane was added and the tube was vortex-mixed for 5 min. The tube was centrifuged for 5 min in a table top centrifuge and the hexane layer, containing the FAME [17], was analysed using GC–MS equipped with an FID detector and using a fused silica capillary column 30 m × 25 mm 1D × 0.2 µm in thickness. The column was initially maintained at 150 °C for 5 min, increased by 12 °C/min to 160 °C and next by 3 °C/min to 220 °C and there it was kept isothermal for 10 min. Injector and detector ports were maintained at 220 °C and carrier gas (nitrogen) pressure was maintained at 18 psi.

Estimation of membrane fluidity

The desired amount of phospholipid sample and 1, 6-diphenyl-1,3,5-hexatriene (i.e. 100:1) was taken in a cryo-vial and dried completely under nitrogen gas. The vial was rehydrated with 2 ml of HEPES buffer, then vortexed and sonicated for 1 min at 37 °C. The above step was repeated twice to obtain small Unilaminar Vesicles (SUV) and the liposome thus prepared was used to study fluidity. Fluorescence polarization of DPH was monitored using a Shimadzu spectrophotometer equipped with polarization accessories. The excitation wavelength was set at 360 nm and emission wavelength at 440 nm with 5 nm slit width. Fluorescence intensities were recorded by keeping the excitation polarizer at 0° with an emission analyser at 0° (LH) and 90° (LV) and again excitation polarization at 90° with an emission analyser at 0° (VH) and 90° (VV) with time programme from 0 to 30 min with an interval of 2 min at 37 °C. Fluorescence anisotropy value was calculated using the formula of Kleinfeld et al. [18].

Estimations of enzyme activities

The activity of HMG-CoA reductase (EC 1.1.1.88) [19] was measured using HMG-CoA as a substrate for catalytic activity, HMG-CoA synthase (EC 2.3.3.10) [20] was measured using acetyl-CoA as a substrate for catalytic activity and glucose-6-phosphate-dehydrogenase (EC 1.1.1.49) [21] was measured using glucose-6-phosphatase as a substrate for catalytic activity in liver, kidney and heart tissues.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences Version 21 (IBM SPSS Statistic). ANOVA followed by Tukey's HSD test and Student's *t* test were performed. The results are presented as mean ± standard error.

Result

Significant difference in the lipid composition of the fish oils from different origins (*Sardinella longiceps, Rastrelliger kanagurta and Clarias batrachus*) was observed. The lipid composition (i.e. the amount of triglyceride (TG), glycolipid, phospholipid, free fatty acid and cholesterol) of the fish oils extracted from *Sardinella longiceps*, *Rastrelliger kanagurta* and *Clarias batrachus* is shown in Fig. 1. TG, glycolipid and PL contents were significantly higher in *Sardinella* compared to *Rastrelliger* and *Clarias* fish oils. The fatty acid composition of these fish oils was previously reported [22, 23]. In brief, all three oils are rich in

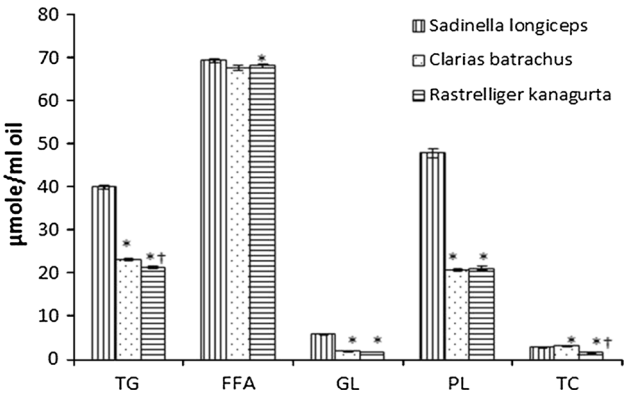

Fig. 1 Lipid composition of fish oil extracted from *Sardinella longiceps*, *Rastrelliger kanagurta* and *Clarias batrachus* (µmole/ml of oil). *TG* triglyceride, *FFA* free fatty acid, *GL* glycolipid, *PL* phospholipid, *TC* total cholesterol. The significant difference between the fish oils based on Student's *t* test and Tukey's HSD test represented by *compared to *Sardinella* oil and †compared to *Rastrelliger* oil

unsaturated fatty acids (43–54 %) including n-3 fatty acids (28–40 %) and n-6 fatty acids (12–15 %). The EPA and DHA contents were 20–25 % higher in the *Sardinella* oil when compared to the other two fish oils.

We have already reported [22, 23] that induction of diabetes results in marked hyperglycaemia with elevated serum free glucose and glycosylated haemoglobin concentrations along with decreased insulin concentration (Table 1). Fish oil supplementation to the diabetic mice significantly decreased glucose and glycosylated haemoglobin concentrations and increased insulin concentration compared with the "control" diabetic mice (Table 1). Due to induction of diabetes, there was also a gradual decrease in body weight nearly by 25 %. However, supplementation of fish oils to the diabetic mice groups prevented the reduction of body weight.

Induction of diabetes elevated the serum TG, TC, VLDL-C and LDL-C concentrations (Table 2), but no significant changes were observed in HDL-C levels. Fish oil supplementation to diabetic animals significantly decreased the levels of TG, TC, VLDL-C and LDL-C when compared with control diabetic mice. Furthermore, HDL-C concentration was increased in the SD and RD groups (Table 2). The liver, kidney and heart tissue lipid profiling showed a significant ($P < 0.01$–0.001) elevation of TG, total glycolipid and TC (Table 3) with a decrease in total PL concentration in diabetic mice. Supplementation of fish oil decreased the elevated levels of TG, total glycolipid and total cholesterol along with an increase in PL levels (Table 3).

The activities of HMG-CoA reductase and HMG-CoA synthase increased significantly ($P < 0.01$–0.05) in diabetic mice, and there was a decrease in the activity of G6PD (Fig. 2). The supplementation of fish oils decreased the activity of HMG-CoA reductase and HMG-CoA synthase and increased the activity of G6PD (Fig. 2).

Assessment of the neutral lipid fraction profile of liver, kidney and heart (Table 3) of diabetic mice showed significant ($P < 0.001$–0.0001) elevation in the concentration of cholesteryl esters, TG, FFA, free cholesterol, di-glyceride along with a significant reduction in the concentration of mono-glyceride when compared with control non-diabetic group mice. In kidney and heart, the concentration of di-glyceride was also increased by diabetes (Table 3). The supplementation of fish oils to diabetic mice decreased the concentration of CE, TG, FFA and free cholesterol and increased the concentration of mono-glyceride when compared to control diabetic group (Table 3). In fish oil-supplemented groups, the concentration of DAG was elevated when compared to both control non-diabetic and control diabetic mice.

The phospholipid profile of liver, kidney and heart tissues showed a significant reduction by 46–57 % in total phospholipid concentration along with a 17–63 % significant ($P < 0.05$–0.001) increase in relative concentration (Fig. 3) of phosphatidylethanolamine, sphingomyelin and lysophospholipid along with a decrease in the relative concentration of phosphatidylcholine and phosphatidylinositol + phosphatidylserine (Fig. 3). We were unable to separate phosphatidylinositol and phosphatidylserine fractions. Supplementation of fish oils to the diabetic group of mice helped to reduce these elevated levels of phospholipid fractions in the liver by 10–32 % along with 15 %−1.1-fold increase in total phospholipid fractions.

The liver, kidney and heart tissues of diabetic mice showed significant ($P < 0.0001$) increase in concentration of glycolipids by 12–40 %. This was also reflected in relative concentration (Fig. 4) of glycolipids fractions i.e. unknown fraction 1 with RI = 0.91 and sphingolipid which was increased by 36 %-2.2 fold along a 21–40 % decrease in glycolipid unknown fraction 2 with RI = 0.32 and galactocerebroside. The supplementation of *Sardinella*, *Clarias* and *Rastrelliger* fish oils to the diabetic group helped recover the alteration in glycolipid concentration. Due to lack of standard, we were unable to identify the glycolipid fractions 1 and 2.

Induction of diabetes in mice was associated with an increase in saturated fatty acid percentage in liver, heart and kidney. The percentage of the saturated fatty acids myristic (14:0), palmitic (16:0) and stearic (18:0) and of the monounsaturated fatty acid palmitoleic (16:1) increased in all three tissues of diabetic mice, while there was a

Table 1 Effect of dietary supplementation of fish oils for 30 days on serum insulin, free sugar and glycosylated haemoglobin level in alloxan-induced diabetic mice (*Mus musculus*). Data represented as a mean of six values and their standard errors

Groups	Insulin (ng/ml)	Free sugar (mg/dl)	Glycosylated haemoglobin (mmol/mol)
NC	0.820 ± 0.011	76.38 ± 11.5	24.35 ± 0.45
ND	0.418 ± 0.010*	566.1 ± 13.8*	155.3 ± 1.62*
SD	0.643 ± 0.011*†	288.1 ± 15*†	64 ± 1.09*†
RD	0.633 ± 0.012*†	290 ± 8.5*†	65.4 ± 0.38*†
CD	0.574 ± 0.014*†‡	298.2 ± 15.3*†‡	69.3 ± 2.6*†

The significant difference between groups for each tissue based on Student's *t* test and Tukey's HSD test represented by * compared to NC, † compared to ND and ‡ compared to SD

Table 2 Effect of dietary supplementation of fish oils on the serum lipid profile in alloxan-induced diabetic mice (Mus musculus)

Parameters	NC	ND	SD	RD	CD
Triglyceride (mg/dl)	131.2 ± 1.1	398.2 ± 1.03*	208.0 ± 1.5*†	214 ± 2.9*†	227.7 ± 4.09*†‡
Total cholesterol (mg/dl)	152.3 ± 0.95	219.1 ± 0.88*	99.76 ± 0.90*†	105.0 ± 0.78*†‡	101.9 ± 0.98*†‡
HDL-C(mg/dl)	22.6 ± 0.99	22.6 ± 0.64	37.3 ± 0.62*†	32.5 ± 0.63*†‡	24.2 ± 0.54‡
VLDL-C (mg/dl)	24.64 ± 1.1	79.6 ± 1.3*	41.5 ± 1.2*†	42.7 ± 1.7*†‡	45.5 ± 1.6*†‡
LDL-C(mg/dl)	102.2 ± 1.8	116.4 ± 2.04*	21.1 ± 2.04*†	29.4 ± 1.3*†	33.4 ± 3.6*†
CH:HDL	6.43	9.80	2.67	3.22	4.21
CH:TG	1.16	0.55	0.479	0.491	0.447

The significant difference between groups for each tissue based on Student's *t* test and Tukey's HSD test represented by * compared to NC, † compared to ND and ‡ compared to SD

decrease in relative percentage of unsaturated fatty acids like oleic (18:1n-9), linoleic (18:2n-6), eicosaenoic (20:1), eicosatrienoic (20:3n-6), arachidonic (20:4n-6), eicosapentaenoic (20:5n-4), docosapentaenoic (22:5n-3) and docosahexaenoic (22:6n-3). The fish oil supplementation (SD, CD and RD groups) increased overall the relative percentage of linoleic (18:2n-6), eicosapentaenoic (20:5n-3), docosapentaenoic (22:5n-3) and docosahexaenoic (22:6n-3) and decreased the oleic (18:1n-9), arachidonic (20:4, n-6) and saturated fatty acid percentage (Table 4).

Anisotropy values for liver, kidney and heart tissue are shown in Fig. 5. An increase in anisotropy reflects more highly constrained motion and lowered fluidity, while a decrease in anisotropy reflects less highly constrained motion and increased fluidity. The results indicate that the fluidity of the membranes in all three tissues is decreased significantly in diabetic mice tissues when compared to control group of mice. Fish oil supplementation returned the fluidity of membrane in liver, kidney and heart tissue towards that seen in non-diabetic animals.

Discussion

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia and deficiency of secretion or action of insulin. In diabetes, numerous complications affecting the vascular system, kidney, retina, lens, peripheral nerves and skin are common and result in increased morbidity and poorer quality of life. Increased oxidative stress is a widely accepted contributor to the development and progression of diabetes and its complications [24, 25]. Several mechanisms, like changes in lipid metabolism [24], imbalance between production of reactive oxygen species and antioxidant defence mechanisms [26] and changes in the inflammatory pathway [27] play an important role in increased oxidative stress in people with diabetes. Much research has demonstrated health benefits of fish in the diet or fish oil supplementation. Several population studies, for example, have suggested that regular consumption of even small amounts of dietary fish may reduce the risk of cardiovascular disease [28, 29], findings supported by one randomized controlled trial [30]. In addition to lowering high blood pressure [29], fish and fish oils rich in omega-3 fatty acids can modify a variety of cellular processes associated with lipid metabolism [31], atherosclerosis, thrombosis and inflammation [32]. These findings suggest a role for n-3 PUFAs in diabetes. The current study focuses on the beneficial effect of three fish oils *S. longiceps*, *C. batrachus* and *R. kanagurta* differing in the lipid and omega-3 fatty acid content of the tissues in diabetes-induced mice. We observed that alloxan-induced diabetes resulted in increased serum TG, TC, VLDL-C and LDL-C levels and decreased HDL level and also elevation in tissue TG, TC and total glycolipid along with changes in neutral lipid composition of tissues. These observations are in agreement with previous reports regarding the alteration in these parameters in the diabetic condition [33, 34]. The lipid abnormalities observed in the type 1 diabetes are mainly due to insulin deficiency. Insulin plays a central role in the regulation of lipid metabolism [35]. In adipose tissue, insulin inhibits the hormone-sensitive lipase. Thus, insulin has an anti-lipolytic action, promoting storage of triglycerides in the adipocytes and reducing release of free fatty acids from adipose tissue into the circulation. The defect in insulin action and hyperglycaemia, or excess mobilization of fat from the adipose tissue because of underutilization of glucose, might be the reason for the lipid abnormalities observed in diabetes [3, 36]. HMG-CoA reductase, which is the rate-controlling enzyme of the mevalonate pathway that produces cholesterol and other isoprenoids, and HMG-CoA synthase, which catalyses the production of HMG-CoA which is an intermediate of cholesterol synthesis, were increased in the tissues of diabetic mice in the present study. The increased activity might be due to prolonged insulin deficiency and exhaustion of substrate for gluconeogenesis and the TCA cycle [37, 38]. The conversion of glucose-6-phosphate to

Table 3 Effect of dietary supplementation of fish oils for 30 days on tissue lipid profile (mg/100 mg) in alloxan-induced diabetic mice (*Mus musculus*)

Parameters	Liver					Kidney				
	NC	ND	SD	RD	CD	NC	ND	SD	RD	CD
Total Triglyceride	2.97 ± 0.22	8.77* ± 0.75	3.23† ± 0.47	3.95*† ± 0.16	3.97*†‡ ± 0.28	2.55 ± 0.26	5.36* ± 0.85	3.07† ± 0.18	3.48* ± 0.10	4.7*†‡ ± 0.72
Total Cholesterol	1.31 ± 0.049	4.36* ± 0.055	2.61*† ± 0.075	2.61*† ± 0.038	3.17*†‡ ± 0.038	1.25 ± 0.029	4.03* ± 0.039	2.10*† ± 0.027	2.47*†‡ ± 0.027	3.08*†‡ ± 0.027
Cholesterol Esters	0.187 ± 0.0013	0.274* ± 0.0021	0.196† ± 0.0033	0.205*†‡ ± 0.0018	0.242*†‡ ± 0.0025	0.162 ± 0.0032	0.265* ± 0.0020	0.171*† ± 0.0015	0.172*† ± 0.0020	0.186*†‡ ± 0.0018
Free fatty acid	1.92 ± 0.099	3.45* ± 0.11	2.11*† ± 0.15	2.13*† ± 0.13	2.42 ± 0.014	1.89 ± 0.12	3.23* ± 0.10	2.02† ± 0.12	2.13† ± 0.12	2.17*† ± 0.15
Free cholesterol	0.155 ± 0.0012	0.230* ± 0.0025	0.108*† ± 0.0022	0.171*† ± 0.0021	0.180*†‡ ± 0.0014	0.144 ± 0.0027	0.223* ± 0.0021	0.152*† ± 0.0026	0.163*†‡ ± 0.0029	0.170*†‡ ± 0.0027
Di-glyceride	0.708 ± 0.075	0.795 ± 0.077	1.3*† ± 0.065	1.11*† ± 0.031	1.065*† ± 0.017	0.676 ± 0.022	0.734* ± 0.030	0.969*† ± 0.023	0.978*† ± 0.034	0.985*†‡ ± 0.032
Mono-glyceride	0.960 ± 0.030	0.447* ± 0.076	3.98*† ± 0.21	3.87*† ± 0.21	2.97*†‡ ± 0.17	0.905 ± 0.016	0.420* ± 0.023	3.33*† ± 0.026	3.25*† ± 0.032	2.41*†‡ ± 0.031

Parameters	Heart				
	NC	ND	SD	RD	CD
Total Triglyceride	3.74 ± 0.48	7.02* ± 0.18	4.08*† ± 0.22	4.62*† ± 0.11	5.36*†‡ ± 0.37
Total Cholesterol	0.676 ± 0.013	1.94* ± 0.031	1.38*† ± 0.011	1.36*† ± 0.023	1.49*† ± 0.021
Cholesterol Esters	0.120 ± 0.0018	0.197* ± 0.0019	0.126*† ± 0.0010	0.125† ± 0.0011	0.148*†‡ ± 0.0011
Free fatty acid	1.74 ± 0.081	3.11* ± 0.12	1.82*† ± 0.10	1.91*†‡ ± 0.14	1.98*†‡ ± 0.15
Free cholesterol	0.126 ± 0.0014	0.196* ± 0.0015	0.125† ± 0.0014	0.134*†‡ ± 0.0022	0.149*†‡ ± 0.0012
Di-glyceride	0.482 ± 0.011	0.560* ± 0.022	0.759*† ± 0.037	0.789*†‡ ± 0.025	0.800*†‡ ± 0.021
Mono-glyceride	0.700 ± 0.011	0.390* ± 0.013	2.79*† ± 0.023	2.54*†‡ ± 0.024	2.28*†‡ ± 0.028

Data represented as a mean of six values and their standard errors

The significant difference between groups for each tissue based on Student's *t* test represented by * compared to NC, † compared to ND and ‡ compared to SD

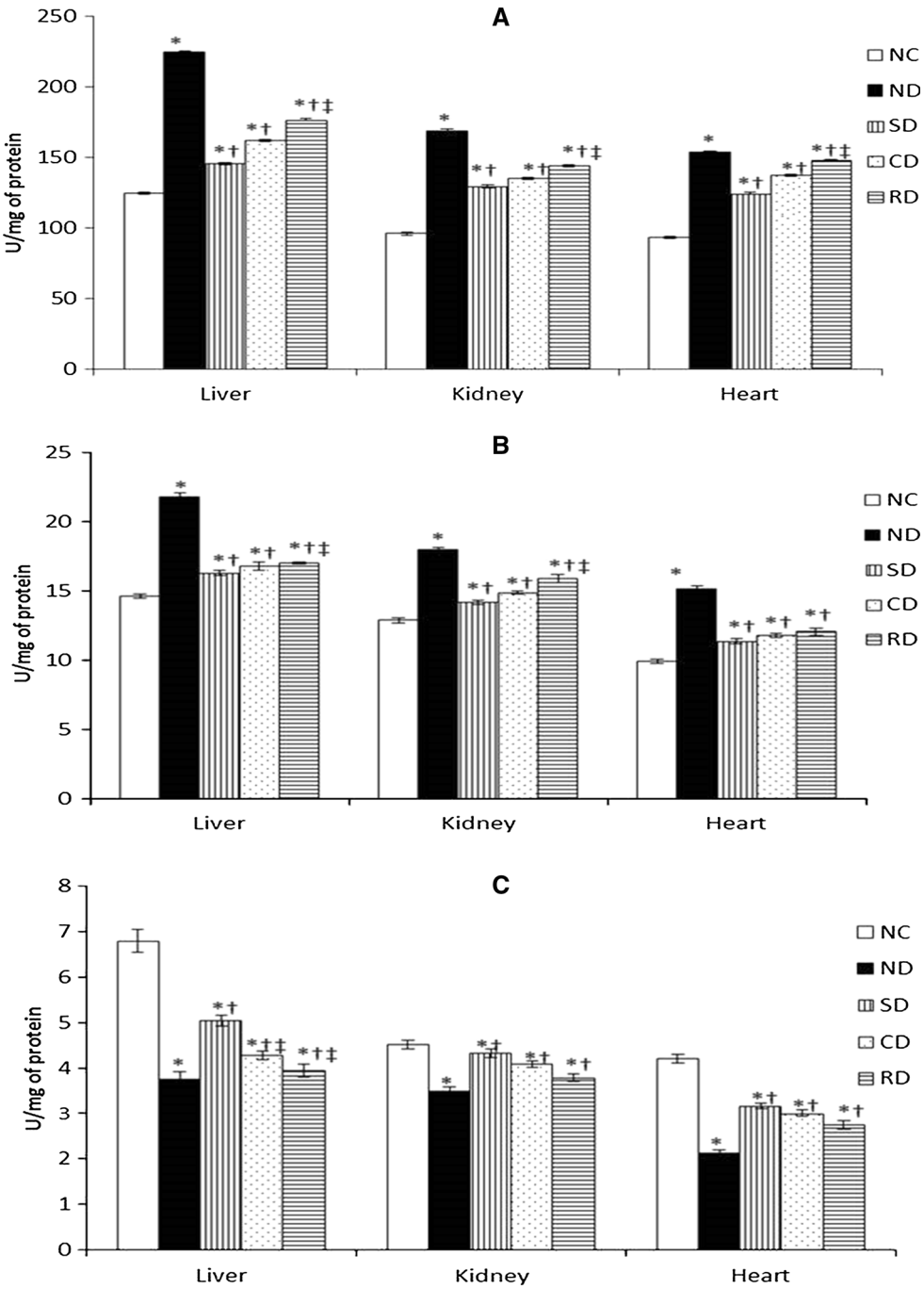

Fig. 2 Effect of dietary supplementation of fish oils for 30 days on the activity of enzymes (U/mg protein) in alloxan-induced diabetic mice (*Mus musculus*). Data represented as a mean of six values and their standard errors. *A* HMG-CoA reductase, *B* HMG-CoA synthase, *C* Glucose-6-phosphate-dehydrogenase. The significant difference between groups for each tissue based on Student's *t* test and Tukey's HSD test represented by *compared to NC, †compared to ND and ‡compared to SD. (*NC* control, *ND* diabetic, *SD* Sardinella oil-supplemented diabetic, *CD* Clarias oil-supplemented diabetic, *RD* Rastrelliger oil-supplemented diabetic mice)

6-phosphogluconate is catalysed by glucose-6-phosphate-dehydrogenase with a simultaneous oxidation of $NADP^+$ to NADPH. This maintains adequate levels of glutathione in its reduced form and helps overcome oxidative stress [39]. We identified a decline in activity of G6PD in diabetic mice which is in accordance with the findings of previous studies which show that the decreased activity of G6PD results in oxidative damage, cellular dysfunction and ultimately organ damage [40–42]. The supplementation of fish oils helped improve the concentration of G6PD in different tissues. The dietary supplementation of fish oil augmented the level of DHA and EPA at the expense of palmitoleic (16:1n-7) and oleic acids (18:1n-9). Augmentation in concentration of phosphatidylethanolamine, lysophospholipid and sphingomyelin along with a decrease in phosphatidylcholine and phosphatidylinositol + phosphatidylserine as observed in diabetic mice shows a positive correlation with a decrease in insulin concentration. This change clearly specifies that the membrane has lost its functional capability, which may arise from alteration in

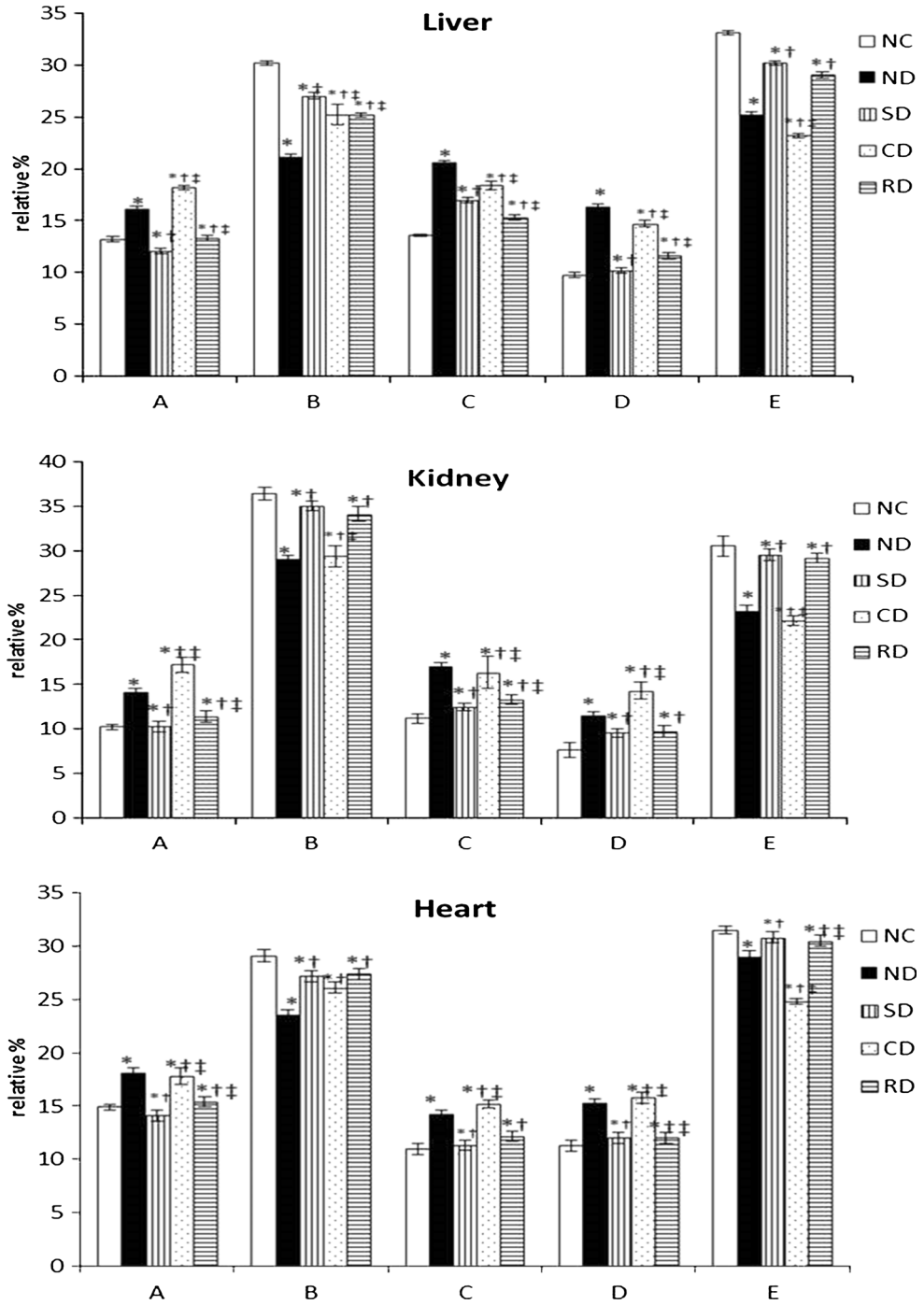

Fig. 3 Effect of dietary supplementation of fish oils for 30 days on tissue phospholipid profile (relative %) in alloxan-induced diabetic mice (*Mus musculus*). Data represented as a mean of six values and their standard errors. *A* Phosphatidylethanolamine, *B* Phosphatidylcholine, *C* Sphingomyelin, *D* Lysophospholipid, *E* Phosphatidylinisitol + Phosphatidylserine. The significant difference between groups for each tissue based on Student's *t* test and Tukey's HSD test represented by *compared to NC, †compared to ND and ‡compared to SD. (*NC* control, *ND* diabetic, *SD* Sardinella oil-supplemented diabetic, *CD* Clarias oil-supplemented diabetic, *RD* Rastrelliger oil-supplemented diabetic mice)

the intake of precursors, alteration in cellular metabolism associated with the biosynthesis and catabolism of the phospholipids [43]. The changes in lipid profile were also reflected in membrane fluidity which is decreased in diabetic mice tissues when compared with control probably due to a higher ordering of phospholipid molecules in the hydrocarbon region of the membranes, i.e. to lowered membrane lipid packing. Also lower PUFA concentration

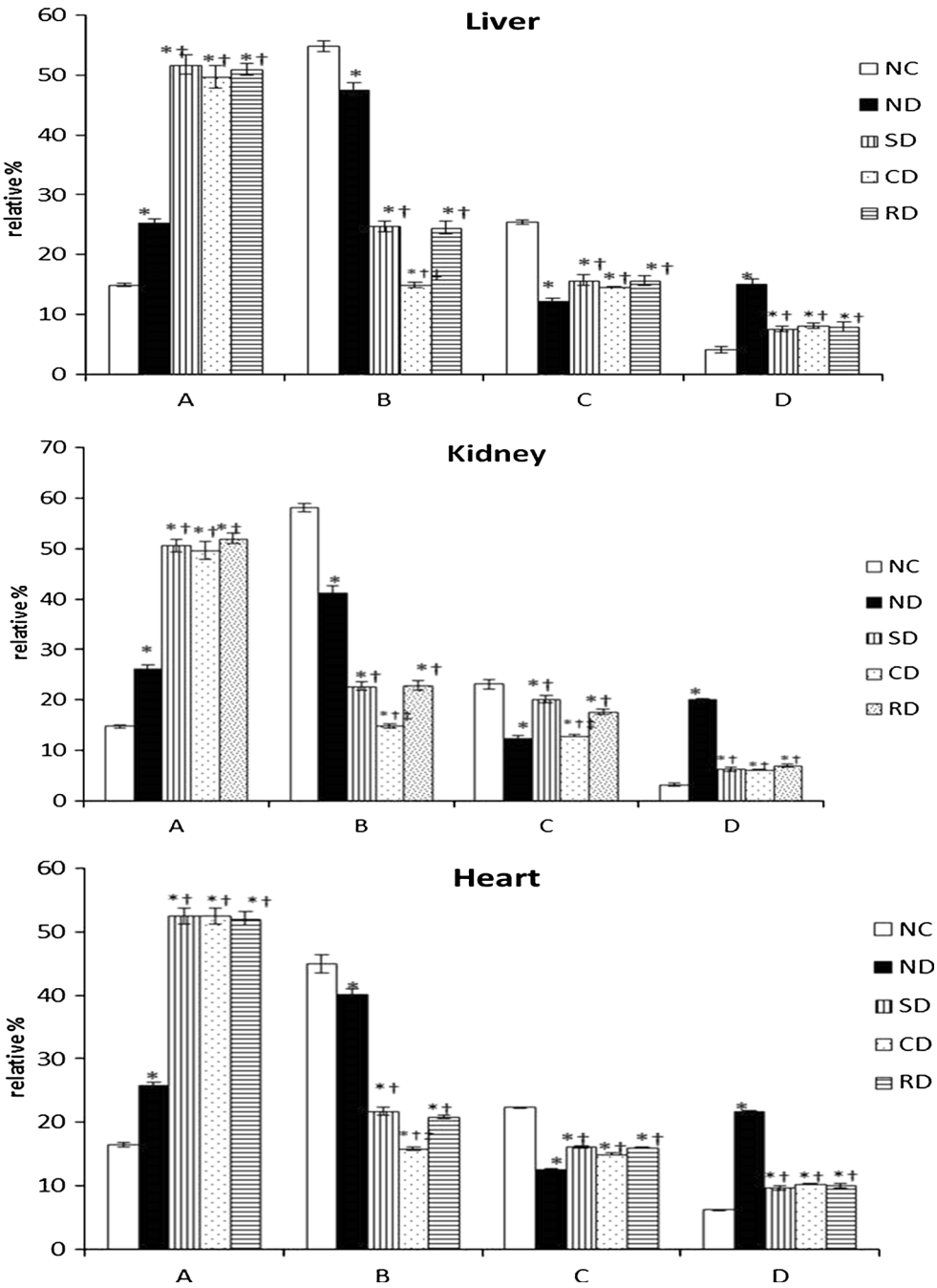

Fig. 4 Effect of dietary supplementation of fish oils for 30 days on tissue glycolipid profile (relative %) in alloxan-induced diabetic mice (*Mus musculus*). Data represented as a mean of six values and their standard errors. *A* unknown 1 (RI = 0.9), *B* unknown 2 (RI = 0.32), *C* Galactocerebroside, *D* Sphingolipid. The significant difference between groups for each tissue based on Student's *t* test and Tukey's HSD test represented by *compared to NC, †compared to ND and ‡compared to SD. (*NC* control, ND diabetic, SD Sardinella oil-supplemented diabetic, *CD* Clarias oil-supplemented diabetic, *RD* Rastrelliger oil-supplemented diabetic mice)

in cell membranes leads to changes of phospholipid fatty acid composition and physicochemical properties of plasma membrane and lowering their fluidity [44]. Our observations are in agreement with previous studies by Waczulíková et al. [45], Bryszewska et al. [46], Watala et al. [47] and Candiloros et al. [48], who observed lowered fluidity in erythrocytes from diabetic patients, along with a significant change in the ordering of phospholipid molecules in membrane lipid bilayer. Supplementation of *Sardinella* oil with higher EPA and DHA contents to the diabetic mice group might help restore the ordering of the phospholipid molecules in the membrane lipid bilayer and the glycolipid composition of extracellular face of cell membrane [49]. The omega-3 PUFA might be responsible for proteolytic effect and regulation of lipid metabolism by regulating key genes SREBP1 and PPARα [50]; however, the exact mechanism of regulation of membrane phospholipid needs to be explored.

Supplementation of fish oils rich in omega-3 PUFAs to diabetic mice helped decrease significantly the level of

Table 4 Effect of dietary supplementation of fish oils for 30 days on liver and kidney tissues' fatty acid profile (area %) of total lipid fraction in alloxan-induced diabetic mice (*Mus musculus*)

Types of fatty acids	Liver					Kidney				
	NC	ND	SD	RD	CD	NC	ND	SD	RD	CD
C14:0	0.55 ± 0.11	1.41 ± 0.058	0.65 ± 0.026	0.84 ± 0.049	1.13 ± 0.061	1.76 ± 0.095	1.80 ± 0.12	1.65 ± 0.05	1.66 ± 0.25	2.26 ± 0.24
C16:0	21.10 ± 0.29	39.31 ± 0.91	29.21 ± 0.55	29 ± 0.76	29.9 ± 1.6	24.1 ± 0.70	30.54 ± 0.86	26.8 ± 0.25	27.47 ± 0.48	28.47 ± 0.36
C16:1(*cis*)	1.06 ± 0.23	2.01 ± 0.20	3.98 ± 0.24	4.17 ± 0.27	3.63 ± 0.15	0.58 ± 0.012	1.03 ± 0.11	2.24 ± 0.16	3.87 ± 0.26	2.56 ± 0.26
C16:1(*trans*)	3.6 ± 0.29	4 ± 0.57	3.11 ± 0.10	3.47 ± 0.30	3.98 ± 0.29	5.7 ± 0.22	5.56 ± 0.31	4.27 ± 0.20	3.17 ± 0.73	5.69 ± 0.34
C18:0	8.63 ± 0.34	11.81 ± 0.64	10.74 ± 0.48	10.91 ± 1.2	11.56 ± 0.16	4.19 ± 0.46	7.21 ± 0.16	5.2 ± 0.58	4.43 ± 0.73	6.27 ± 0.60
C18:1(*cis*)	25.8 ± 2.26	19.02 ± 0.58	16.39 ± 0.94	17.21 ± 1.3	12.53 ± 0.9	32.10 ± 0.58	26.16 ± 0.40	28.92 ± 2.9	29.7 ± 1.01	26.21 ± 0.35
C18:1(*trans*)	8.09 + 0.30	4.58 ± 0.6	5.54 ± 0.80	3.23 ± 0.62	7.85 ± 1.1	4.01 ± 0.21	5.16 ± 0.36	5.1 ± 1.9	3.29 ± 0.15	4.05 ± 0.44
C18:2(n-6)	6.37 ± 0.37	4.52 ± 0.54	7.77 ± 0.12	7.4 ± 1.39	6.63 ± 0.29	7.02 ± 0.41	6.42 ± 0.32	6.81 ± 0.43	8.2 ± 0.32	8.95 ± 0.73
C20:1	0.93 ± 0.031	0.46 ± 0.12	0.63 ± 0.034	0.82 ± 0.19	0.85 ± 0.053	0.78 ± 0.017	1.7 ± 0.64	0.43 ± 0.11	1.33 ± 0.11	2.63 ± 0.11
C20:3(n-6)	1.07 ± 0.018	0.67 ± 0.21	1.97 ± 0.018	1.72 ± 0.10	2.37 ± 0.51	0.74 ± 0.095	1.45 ± 0.19	1.18 ± 0.29	1.44 ± 0.20	0.67 ± 0.13
C20:4(n-6)	9.34 ± 0.016	3.95 ± 0.4	2.42 ± 0.041	2.51 ± 0.29	2.48 ± 0.56	7.61 ± 0.46	5.11 ± 0.24	4.2 ± 0.81	2.67 ± 0.19	2.31 ± 0.052
C20:5(n-3)	4.61 ± 0.055	2.39 ± 0.061	9.05 ± 0.30	9.20 ± 0.67	8.98 ± 0.48	4.81 ± 0.70	2.25 ± 0.49	7.57 ± 1.3	6.31 ± 0.23	5.00 ± 0.31
C22:5(n-3)	6.8 ± 0.86	5.05 ± 0.56	6.53 ± 0.69	6.05 ± 0.63	5.85 ± 0.30	4.03 ± 0.28	3.16 ± 0.30	2.79 ± 0.12	3.61 ± 0.92	2.91 ± 0.71
C22:6(n-3)	1.31 ± 0.33	0.81 ± 0.058	2.01 ± 0.104	2.04 ± 0.44	2.25 ± 0.41	2.57 ± 0.44	2.15 ± 0.075	2.55 ± 0.22	2.20 ± 0.11	2.02 ± 0.25
Saturated	30.28	52.53*	40.60*†	40.75*†	42.59*†	30.05	39.55*	33.65*†	33.56*†	37*†
Unsaturated	69.72	47.47*	59.4*†	59.25*†	57.4*†	69.95	60.15*	66.06*†	65.79*†	63*†
n-3	12.72	8.25*	17.59†	17.29†	17.08†	11.41	7.56*	12.91*†	12.12*†	9.93*†
n-6	16.78	9.14*	9.14†	11.63†	11.48†	15.37	12.98*	12.19†	12.31†	11.93*

Types of fatty acids	Heart				
	NC	ND	SD	RD	CD
C14:0	1.66 ± 0.063	2.63 ± 0.44	1.82 ± 0.040	2.03 ± 0.090	2.01 ± 0.24
C16:0	24.48 ± 0.42	28.46 ± 0.84	20.14 ± 0.48	22.43 ± 0.53	25.07 ± 0.59
C16:1(*cis*)	1.13 ± 0.24	2.13 ± 0.19	2.11 ± 0.10	3.52 ± 0.24	3.53 ± 0.34
C16:1(*trans*)	5.86 ± 0.34	5.28 ± 0.49	6.34 ± 0.16	6.03 ± 0.48	6.96 ± 0.64
C18:0	8.05 ± 0.76	12.85 ± 1.14	15.1 ± 0.10	12.84 ± 0.65	12.88 ± 0.33
C18:1(*cis*)	25.6 ± 1.89	20.04 ± 0.97	20.79 ± 1.09	19.8 ± 1.1	18.47 ± 1.1
C18:1(*trans*)	5.33 + 0.45	4.42 ± 0.23	4.22 ± 0.46	4.01 ± 0.51	2.61 ± 0.41
C18:2(n-6)	9.73 ± 0.28	6.58 ± 0.30	7.79 ± 0.17	7.33 ± 0.43	7.24 ± 1.0
C20:1	1.32 ± 0.18	1.16 ± 0.29	1.60 ± 0.13	1.86 ± 0.061	1.78 ± 0.071
C20:3(n-6)	1.0 ± 0.12	1.74 ± 0.095	1.05 ± 0.027	1.33 ± 0.085	1.70 ± 0.18
C20:4(n-6)	6.4 ± 0.26	5.11 ± 0.51	4.22 ± 0.49	3.51 ± 0.46	2.34 ± 0.44
C20:5(n-3)	3.14 ± 0.12	2.54 ± 0.11	6.43 ± 0.14	5.63 ± 0.42	5.44 ± 0.49
C22:5(n-3)	2.06 ± 0.33	4.91 ± 0.95	5.19 ± 0.26	7.13 ± 0.49	7.69 ± 0.58

Table 4 continued

Types of fatty acids	Heart				
	NC	ND	SD	RD	CD
C22:6(n-3)	4.24 ± 0.30	1.36 ± 0.047	3.20 ± 0.096	2.15 ± 0.075	1.38 ± 0.21
Saturated	34.19	43.94*	37.06*†	37.3*†	39.96*†
Unsaturated	65.81	55.27*	62.4*†	62.3*†	59.14*†
n-3	9.44	8.81*	14.82*†	14.91*†	14.51*†
n-6	17.13	13.43*	13.06†	12.17†	11.28*

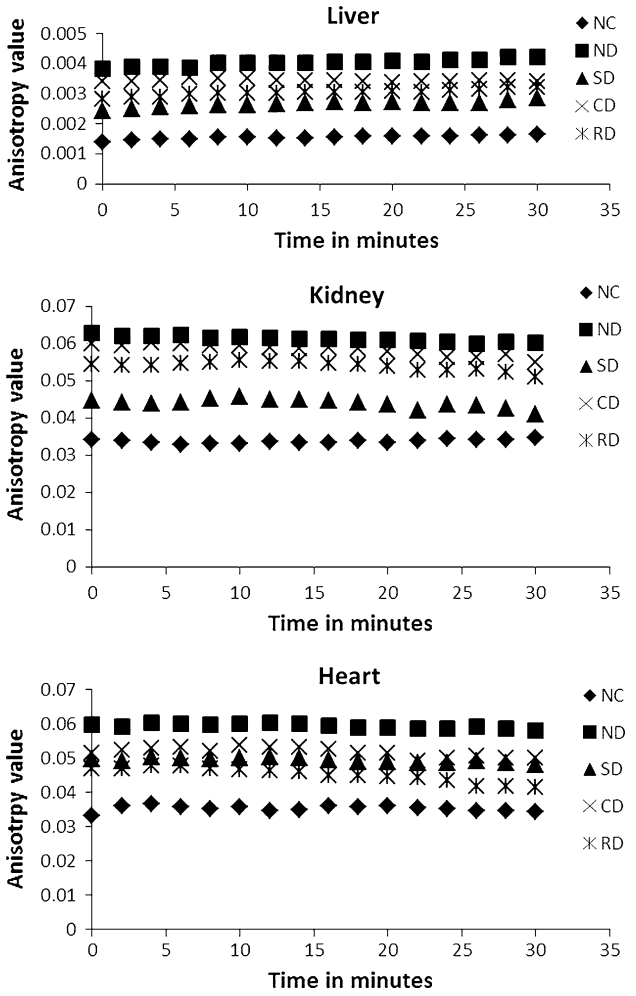

Fig. 5 Effect of dietary supplementation of fish oils for 30 days on the membrane fluidity of tissues in alloxan-induced diabetic mice (*Mus musculus*). *NC* control, *ND* diabetic, *SD* Sardinella oil-supplemented diabetic, *CD* Clarias oil-supplemented diabetic, *RD* Rastrelliger oil-supplemented diabetic mice

serum TG, TC, VLDL-C and LDL-C and tissue TG, TC and total glycolipid along with an increase in level of serum HDL-C and tissue phospholipid concentration. The supplementation of fish oils also helped recover the changed neutral lipid profile of the tissues as shown by our results. Omega-3 PUFAs have an important triglyceride-lowering effect that has been mainly ascribed to reduce hepatic TAG synthesis and VLDL assembly [51]. Omega-3 fatty acids might suppress hepatic lipogenesis and reduce circulating TG levels as a result [52]. Omega-3 PUFAs can also depress cholesterol synthesis and may reduce cholesterol absorption [53, 54] through down-regulation of the hepatic LDL receptor, but the mechanisms by which these fatty acids regulate HMG-CoA reductase and synthase are unclear [55]. The membrane fluidity index depends on two major factors: the level, composition and percentage of PUFA in the membrane and level of membrane cholesterol.

An increase in PUFA results in fluidizing the membrane, while an increase in cholesterol hardens the membrane by decreasing fluidity [56]. The increase in membrane fluidity in fish oil-treated diabetic mice compared with control diabetic mice may be due to the n-3 PUFAs present in fish oil getting incorporated into cellular membranes, where they can mediate their effects by restructuring of membrane structure, increasing membrane fluidity and altering the subcellular localization of proteins [57].

The actions of omega-3 PUFA present in fish oil appear to involve in multiple mechanisms that connect the cell membrane, the cytosol and the nucleus. PUFA acts via eicosanoid receptors which are integral membrane proteins that detect the presence of signalling molecules. Fish oils containing EPA might act as the natural COX inhibitor by inhibiting AA metabolism and acting as an alternative substrate for COX and is the main reason of improved lipid and lipoprotein metabolism [58]. However, the further work is required to understand clearly the mechanism of action of fish oil.

In conclusion, our study has demonstrated that the fish oils rich in omega-3 fatty acids especially EPA and DHA are able to significantly improve lipid metabolism and membrane fluidity of diabetic mice. This supports the concept that dietary fish oils rich in omega-3 PUFA may be of therapeutic benefit in patients with diabetes.

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Compliance with ethical standards

Conflict of interest The study was carried out in strict accordance as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals, Govt. of India). The authors declare that they have no conflicts of interest concerning this article.

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