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Original Article

Beneficial effect of Omega-3 polyunsaturated fatty acids on neurosensorial impairments and oxidative status in Streptozotocin induced diabetic rats

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Abstract

Objectives: The present study evaluated the beneficial effects of omega 3 PUFA present in fish oil on neurosensorial impairments, namely learning, memory and anxiety in Streptozotocin (STZ) induced diabetic rats. The brain homogenate was analysed for oxidative status following Omega 3 PUFA treatment.

Methods: Male, Wistar rats of 2-3 months old were divided into non diabetic controls, diabetic control, & fish oil treated diabetic rats (n=6). Diabetes was induced by injection of STZ (48 mg/kg, ip). Animals were treated orally for 30 days with a dose in each group of 0.5 g/kg/day of fish oil. All experiments were conducted after ethical committee clearance was obtained.

Results: Memory and exploratory behavior were improved ($p<0.01$) in fish oil treated rats as compared with diabetic rats. A significant ($p<0.001$) decrease in MDA and a significant increase ($p<0.001$) in total antioxidant level (TAO) were observed in fish oil treated rats.

Conclusion : Omega 3 PUFA present in fish oil could be used an adjuvant therapy for treatment and prevention of neurosensorial impairment in diabetes mellitus.

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Introduction

Diabetes mellitus is a cluster of abnormal metabolic disorders. There are substantial number of clinical and experimental evidences associating diabetes

mellitus with neurosensorial impairment and neurodegeneration in the hippocampus (1-3). However multifactorial pathogenesis of cognitive impairment in diabetes has not been fully elucidated till today. The underlying mechanism of cognitive impairment in diabetes have been associated with high glucose level, lack of insulin, increased oxidative stress, hyperactivity of Hypothalamo Pituitary Axis and activation of inflammatory pathways (4-6). Studies have revealed that longlasting diabetes and hyperglycemia induced using streptozotocin in rat models could be associated with a decrease in cerebral concentration of serotonin and with an accompanying increase in the number of 5-HT(1A) and 5-HT2 receptors in the brain areas (7). It has been suggested that this change in serotonergic transmission in the CNS could play a role in diabetes-related behavioural abnormalities.

A role for dietary supplementation of Omega 3 PUFA has been under intense investigation recently. Detailed trials have been conducted in older subjects by providing them food supplements with PUFA (8) in order to investigate the improvements in cognitive capability. However the studies on US soldiers deployed in Gulf war showed no significant improvement in mood and cognitive functions following treatment with omega 3 PUFA at a dosage of 2.5 g/ day, even though there was an increase in neurophysiological parameters (9). Further, current clinical trials are of the opinion that PUFA containing micronutrient preparations could improve cognitive functions and have beneficial effects in degenerative diseases such as Alzheimer's disease (10).

Polyunsaturated fatty acids are essential for lipid derived modulators of cell signalling, gene expression and inflammatory process, as these are not synthesized in the human body, they need to be supplemented through the diet (11, 12). Omega 3 PUFA such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and alpha-linolenic acid (ALA) are essential for the neural growth and development in mammals. They facilitate the formation of dendritic spines (13), activate the anti-apoptotic pathway and significantly improve the ability of learning and memory (14). Omega 3 PUFA reportedly protected glutaminergic neurotransmission from stress induced damage, and possibly preventing the development of stress-related disorders such as depression or anxiety. Further they suggested that hippocampus contains glucocorticoid receptors and is involved in learning and memory (15).

It has also been proved that in olfactory bulbectomised rats, fish oil increases the hippocampal levels of brain-derived neurotrophic factor (BDNF) and serotonin (5-HT), the two major regulators of neuronal survival and long-term plasticity in these brain structures (16). Elevated oxidative stress in the brain tissues of the diabetics could be one of the major molecular mechanisms responsible for the pathogenesis and progression of neurodegenerative changes (17).

Diabetic neurodegneration has been a clinical problem, which needed special attention. Dietary supplementation with easily available substances could be highly useful in containing deleterious effects of diabetes mellitus. Thus considering the potential modulatory effects of PUFA supplementation on cognition, anxiety like behaviour and brain antioxidant status we hypothesize that a supplementation of Omega-3 PUFA i.e. DHA and EPA present in the fish oil could be useful in reversal of neurosensorial impairment in streptozotocin diabetic rats. The results suggest unequivocal beneficial effects of the nutritional supplementation among the diabetic rats.

Materials and methods

Animals

Healthy two-three months old inbred Wistar strain albino male rats of body weight 200-250 grams were taken for the study. Animals were maintained according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experts on Animals (CPCSEA), Government of India. Approval of Institutional Animal Ethical Committee (IAEC) was obtained before the experiments were started (letter dated 2/02/2013). Animals were housed individually; food and water were available ad libitum.

Induction of diabetes

Animals were administered with a single intraperitoneal injection of STZ (48 mg/kg) dissolved in 10 mM sodium citrate buffer (pH 4.5) animals of the control groups received single i.p. and injection of the buffer alone. These rats were supplied with 5% glucose solution for next 12 hours in order to prevent acute hypoglycemia. On 3rd and 10th day following the administration of STZ, blood glucose levels were measured using blood glucometer (*Rite Check, Mediplus, India.)* to confirm stable hyperglycemia. Animals with blood glucose levels above 300 mg/dl were selected for the study and kept in constant hyperglycemic state for 20 days.

Fish oil

Fish oil was procured from a reputed company (*NOW omega-3)* through online from http:// www.healthkart.com, the composition was: Energy 10.32 Kcal; Carbohydrate 0.19g; Protein 0.24 g; Fat 0.95 g; d-Alpha Tocopherol 30.00IU; Salmon Oil 1030.50 mg; EPA (Eicosapentaenoic acid) 180.00 mg; DHA (Docosahexaenoic acid) 120.00 mg. The fatty acid composition of a single fish oil softgel capsule was 500mg which includes EPA (18%) and DHA (12%). Based on previous literature (18) a low dose of 0.5 g/kg/day of omega 3 PUFA was given orally using oral feeding gauge between 9-10AM everyday for 30 days.

Preparation of brain tissue homogenates

Rat brain was removed from the skull and was cut into small pieces using rat brain slicer (Zovic, Germany). Brain tissue was weighed and homogenization was carried out in ice cold Phosphate saline buffer (PBS, pH 7.4) to yield a 10% (w/v) tissue homogenates. The supernatant solution was collected and stored at –80°C until used.

Behavioral tests

Open-field exploration (OFT) was used to evaluate the anxiolytic activity and Passive avoidance test was done to assess the memory. All the behavioral tests were performed on completion of 30 days of oral administration of fish oil.

Open-field exploration test

Open field test is one of the most widely used method to assess the motor, exploratory activities and emotional reactivity of rodents (19). The animal was placed in a brightly lit box (100x100x40 cm) with a floor consisting of 25 squares. Illumination was provided by 100 watt bulb fixed at 60 cm above the centre of the field as described by Bures et al. (19). Each animal was placed in the open field apparatus during which theparameters quantified were: ambulation, rearing, grooming, activity in centre and fecal droppings.

Passive avoidance test

Passive avoidance apparatus consists of a wooden box of bright and dark compartment with grid floor, which is attached to a shock source. On the first day of the test rats were allowed to explore both chambers for five minutes. This was followed by three test trials of five minutes each. In each trial, fraction of time spent in each compartment was measured. In 4th trial, as soon as the rats stepped into the dark compartment, a foot shock (2.5 mA) was given and rats were returned to home cage (20). After 24 hours, rats were placed in the test chamber and latency to enter the dark compartmentwas measured.

Biochemical estimations

Malondialdehyde (MDA) : Malondialdehyde (MDA) was assessed for Lipid peroxidation in the brain tissue by applying the method as per Ohkawa, Ohishi and Yagi (21) using thiobarbituric acid (TBA) reagent. MDA formed by the breakdown of polyunsaturated fatty acids serves a convenient index to determine the extent of lipid peroxidation. It reacts with TBA to give a pink color which is read by spectrophotometer at 532 nm (Systronic 117).

Total antioxidants (TAO): Total antioxidant capacity was estimated from the brain tissue homogenates by Koracevic, et al. 2001 method (22) which is based on the suppression of the formation of thiobarbituric acid-reactive substances (TBARS) by the antioxidants in brain tissue homogenate. This reaction was measured spectrophotometrically at 532 nm.

Statistical analysis

Analysis of the data was done using the statisticals of tware SPSS 16th version; data was expressed as mean±SEM. Level of significance was fixed as 5%. One way ANOVA was done to compare the biochemical parameters and open field test parameters in between the different groups. Intergroup comparison was done by post hocTukey's test. Passive avoidance test parameters and MDA levels were analyzed by Kruskall Wallis Test as they did not fall the normal distribution. Graphs were done by using Graph Pad Prism 6 Demo (GraphPad Software IC., CA, USA).

Results

Open field test

There was significant difference between the groups as determined by One Way ANOVA in the following parameters assessed by the open field test; a. Number of peripheral squares crossed ($F(2,15) = 18.971$, $p<0.001$), b. Number of central squares crossed ($F(2,15) = 4.895$, $p<0.05$,) and c. Rearing score ($F(2,15) = 8.102$, $p<0.01$). There was no statistically significant change in grooming score ($F= (2, 15) = .328$, $p=0.726$) and defecation score ($F(2, 15)$ $p<0.01$, $p=0.988$).

Post hoc test by Tukey's test indicated the mean score for the number of peripheral squares entered by fish oil treated group was significantly ($p<0.001$) higher than the diabetic control, whereas diabetic control had significantly ($p<0.001$) lower number of peripheral squares entered as compared to the normal control. There was no significant difference between normal control and fish oil treated rats ($p=.151$).The mean score for the number of central squares entered by diabetic control was significantly ($p<0.05$) lower than the normal control. Whereas there was no significant difference in fish oil treated group and normal control and also between diabetic control and fish oil treated rats ($p=.952$). The number of rearing by diabetic control was significantly ($p<0.01$) lower than the normal control. Where as no significant difference was seen between normal control and fish oil treated rats as well as between diabetic control and fish oil treated rats (Table I).

Passive avoidance test

By Kruskall Wallis test, there was a statistically significant ($p<0.05$) change in the latency to enter the dark chamber on the second day among the groups. Post hoc by Tukey's was observed test showed that there was a significant difference between normal control and diabetic control, where as there was no significant change between fish oil and diabetic control group and also between fish oil andnormal control (Fig. 1).

Brain tissue MDA level

Kruskall Wallis test showed statistically significant ($p<0.001$) difference between the groups. Post hoc comparison using the Tukey's HSD test indicated that MDA level of fish oil treated group was significantly ($p<0.001$) lower than the normal control and also the diabetic control ($p<0.001$). There was

TABLE I: Effect of Fish oil on open field test in rats.

Group (n=6)	*Peripheral squares (n)*	*Central squares (n)*	*Rearing (n)*	*Self grooming (n)*	*Defecation score (n)*
Group I (Normal control)	37.5±4.9	6.33±2.6	11.33±1.3	5.16±2.4	3.0±0.73
Group II (Diabeticcontrol)	4.5±1.1***	.0000±.00*	2.83±1.1**	3.66±1.3	3.16±2.3
Group III (Fish oil treated)	26.6±4.3**	.66±0.6	8.16±1.8	5.33±0.3	3.0±2.1

One Way ANOVA followed by Turkey's test, ***p<0.001, **p<0.01, *p<0.05, Values are Mean±SEMNS - Nonsignificant

Peripheral Square	Central Square	Rearing	Grooming	Defecation
Grp I-GrpII: p=0.000	Grp I-GrpII: p=0.031	GrpI-GrpII:p=0.003	Grp I-GrpII: NS	Grp I-GrpII: NS
GrpII-Grp III: p=0.003	GrpII-Grp III: NS	GrpII-Grp III: NS	GrpII-Grp III: NS	GrpII-Grp III: NS
GrpI-Grp III: NS	GrpI-Grp III: NS	GrpI-Grp III: NS	GrpI-Grp III: NS	GrpI-Grp III: NS

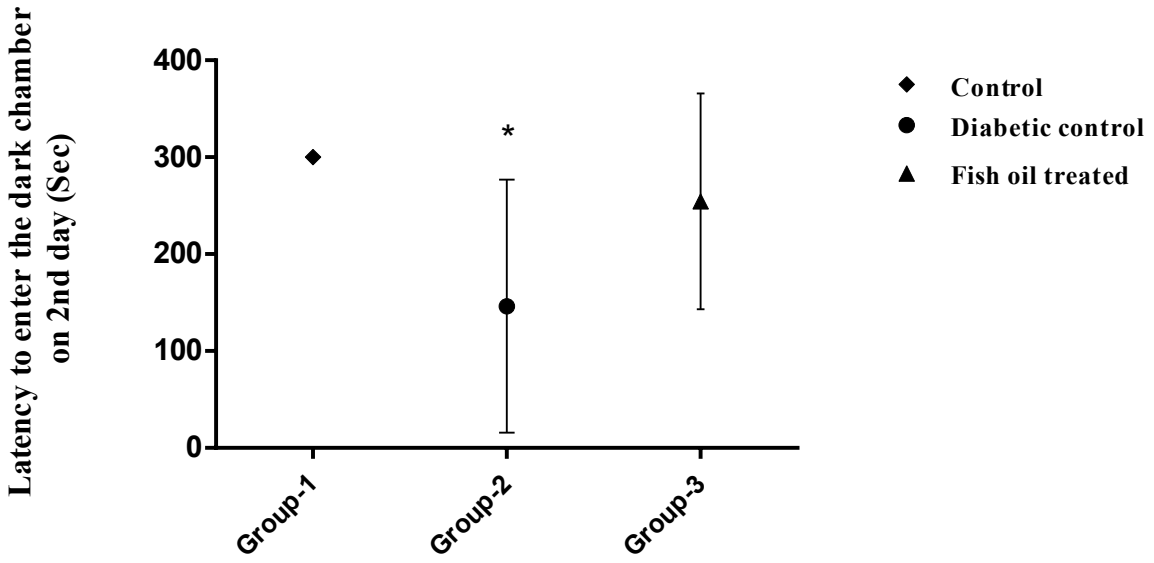

Fig. 1 : Effect of fish oil treatment on the latency to enter the dark chamber on second day of passive avoidance test. Kruskall Wallis test ($p<0.05$) followed by Tukey's test was done for multiple comparisons *$p<0.05$.

no statistically significant difference of MDA level between diabetic control and normal control rats ($p=0.842$) (Table II).

TABLE II : Effect of fish on Malondialdehyde (MDA) levels in different groups.

Group (n=6)	*MDA (µmole/L)*
Group I (Normal control)	2.69 (2.47,2.72)
Group II (Diabetic control)	2.92 (2.52,2.98)***
Group III (Fish oil treated)	0.914 (0.853,0.987)***

Brain tissue total antioxidants level

There was statistically significant difference between groups as determined by one-way ANOVA ($F(2, 15) =21.97$, $p<0.001$).Tukey's post-hoc test revealed that total antioxidant activity in the brain tissue of the fish oil treated diabetic group had significantly ($p<0.001$) higher total antioxidant activity as compared to the diabetic control. Whereas diabetic control had significantly ($p<0.001$) lower total antioxidant activity than the normal control. There was no significant difference of total antioxidant capacity between normal control and fish oil treated rats ($p=.235$) (Table III).

TABLE III : Total Antioxidant activity (TAO) in different groups.

Group (n=6)	*TAO (mmol/L)*
Group I (Normal control)	5.9783±0.3
Group II (Diabetic control)	4.15±0.31***
Group III (Fish oil treated)	6.64±0.17***

One way ANOVA test followed by Tukey's test, $F=21.975$, $p=0.000$, ***$p<0.001$; Data as mean±SEM; Grp I - Grp II: $p<0.001$, Grp II - Grp III: $p<0.001$, Grp I - Grp III: Not significant.Kruskall Wallis test followed by Tukey's test, Data expressed as median (25^{th} quartile, 75th quartile).

Grp I - Grp II: Not significant, Grp II - Grp III: $p<0.001$, Grp I - Grp III: $p<0.001$, ***$p<0.001$.

DISCUSSION

In the present study we aimed to investigate the protective effects of fish oil (Salmon fish oil) on the neurosensorial impairments and oxidative stress in STZ induced diabetic rats. Cognitive deficit has been established in the STZ induced diabetic rats in the present study (Fig. 1). A reduction in sensorimotor activity in the untreated diabetic rats is evident from the open field task results, which are in compliance with the previous study (23). Fish oil treatment significantly ($p<0.01$) improved open field activity in diabetic rats. This observed anxiolytic activity of omega 3 PUFA in diabeticrats could be explained

on the basis of augmentedserotonin level by fish oil supplementation (16). The Salmon oil treated group showed a significant improvement compared to untreated group, and the scores of passive avoidance test among control and fish oil treated group was comparable. The memory retention showed similar trend ($p<0.05$) by the latency to enter the dark chamber on the second day of passive avoidance between the normal control group and diabetic rats, the results fall in agreement with the previous study (24). Beneficial effect of fish oil was further evident in memory retention, as evidenced in the delay in entering the dark chamber by the fish oil treated diabetic rats on the second day after having experienced shock on the previous day.

It has been now well established (25, 26, 27) that psychologicalrisk factors, such as depression and anxiety are independently associated with different forms of diabetes and also an increased risk of socio-economic problemsand morbidities and mortalities (28). Our study further emphasises that Fish oil could prove an useful dietary supplement in improving depression in diabetes (29). However a definitive conclusion is yet to be made on the complexity of the cause - effect relationship between diabetes and diverse types of neurosensorial impairments. Controversy still exists on the food regimen for diabetic patients. In view of this situation it is reasonable to suggest that at present omega 3 PUFA richly available in fish oil could aswell be plausible and feasible therapeutic alternativefor diabetic patients with neurosensorial impairment. Though the underlying pathophysiology of neuronal deficit in diabetes yet to be understood, Streptozotocin-induced diabetic ratshave shown a significant inhibition of serotonergic functions in different brain regions (7) which gets reversed on insulin replacement therapy.

Reports have demonstrated that increased microvascular disorders and oxidative stress due to Reactive Oxygen Species (ROS) produced as a result of diabetes mellitus which play an important role in the development and prog ression of diabetic complications in the brain (27, 30). Damage due to oxidative stress to various brain regions contributes to its morphological abnormalities and memory impairment (31). Antioxidants likeCurcumin(32), melatonin andvitamin E(33) have been shown to protect neurons against a variety of experimental neurodegenerative conditions thus preventing diabetes induced learning and memory deficit. In this experiment fish oil administration significantly reduced the MDA levels in the whole brain tissue homogenates which has been reported by previous studies also (34). Further, TOA was increased in the treated animals in our experiments. This suggested a strong evidence for the beneficial effects of fish oil supplementation as an alternative/additive diet for the diabetic patients. Omega 3 PUFA present in the fish oil might have made the neural tissues less susceptible to lipid peroxidation leading to the beneficial effects (35) and it is a source of naturally available antioxidant. Further Omega-3 PUFA was reportedly necessary for hippocampal brain derived neurotrophic factor (BDNF) and activation of the NR2B subunit of the N-methyl-d-aspartate (NMDA) receptor (36) which are essential for cognitive functions, these findings were in line with our findings.Neural tissues are poor in antioxidant enzymes which emphasises the importance of increasing their levels for the primary defence against free radical injury caused by hyperglycemia. In view of this situation it is reasonable to suggest that omega 3 PUFA richly available in fish oil could be a feasible therapeutic supplement for diabetic patients with neurosensorial impairment. Cosar Met al. conducted study on the use fish oil starting from third day of induction of diabetes and found that the hippocampal function was reportedly improved after 8 weeks of treatment, further strengthens our finding of improvements in neurosensoral parameters (34). A positive physiological effect on cognitive parameters and oxidative status was evident at this low dose. Hence dietary supplementation of fish oil could be an adjunct therapy for the prevention and treatment of cognitive impairment in diabetes mellitus. It has been recently reported in journal nutrition that fish eating improved cognitive performance in older people (37).

From this study we confirm that there was decline in sensorineuronal faculty in diabetic animals. Supplementation with Omega-3 PUFA for a period of 30 days (leaving 20 days of untreated diabetic status) showed a definite improvement in the test parameters, suggesting a beneficial effect. Therefore it can be recommended that Salmon fish oil could be added to the dietary regimen of chronic diabetic patients.

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