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Omega-3 PUFA As Food Supplementation Improves Performance of Cognitive Parameter in Type 1 Diabetes Mellitus Model of Wistar Rats.

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ABSTRACT

In Diabetes Mellitus (DM) chronic degenerative brain changes is characterized by impaired cognition, abnormalities of neurochemistry and neuronal structure. This study was conducted to evaluate the effect of dietary supplementation of omega 3 PUFA on cognitive parameters in Streptozotocin (STZ) induced diabetic rats. Adult male Wistar rats were divided into six groups- normal control, diabetic control & four different treated groups (N=46). Diabetes was induced by intraperitoneal injection of STZ (48 mg/kg). Omega 3 supplementation in the form of fish oil and flaxseed oil for 30 days at two different doses (0.21g/kg BW as low dose and 0.5g/kg BW as high dose). Cognitive parameters were assessed by Morris Water Maze (MWM), Passive avoidance test and Open field test. There was significant increase in memory retention in treated groups compared to diabetic control group in passive avoidance test ($p<0.05$) and MWM. There was significant ($p<0.05$) change is acquisition trial results of MWM. Anxiolytic effect of omega 3 was evident from the open field test results. By preventing the early onset of neuronal degeneration in diabetic we can improve the quality of life for these large mass of diabetic people. Our study provides an adjunct therapy for this neuronal degeneration at an affordable price for all the people with different food habits.

Keywords: Omega 3, Diabetes Mellitus, Cognition, Streptozotocin,

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INTRODUCTION

Diabetes mellitus (DM) has become one of the major health challenges of 21st century, with the number of affected people estimated to reach up to 552 million by 2030 (1).Be it a rich or poor country none of them are going to be impervious from this chronic, incurable but preventable Non communicable disease (NCD) (2).Cognitive impairment and high glucose levels are positively correlated (3),probably mediated by structural changes in learning-relevant brain areas (4).Pathophysiology of cognitive impairment in DM can be attributed to a wide range of metabolic and vascular disturbances (5)but the exact mechanism by which DM affects the brain remains unclear. Constant hyperglycemic state in DM is associated with gradual end-organ damage in the central nervous system (6).This little-known complication, referred to as 'diabetic encephalopathy', is characterized by some biochemical alterations such as direct glucose toxicity in the neurons due increased formation of advanced glycation end products which leads to an increase in reactive oxygen species production (7,8), thus overwhelming the antioxidant defence system leading to neuronal cell and cerebrovascular damage (9). Diabetes mellitus induces the apoptosis of CA1 pyramidal neurons, which may be the main contributory factor leading to the deficits in performing the spatial learning task (10).Till date there are no specific pharmacological treatments for reducing or preventing cognitive impairment in patients with DM. In this scenario dietary supplementation of omega 3 PUFA brings a ray of hope for the millions of people suffering with diabetes induced cognitive dysfunction. Omega 3 PUFA like eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and α-linoleic acid (ALA) supplementation may prevent the cognitive decline by maintaining membrane fluidity, increasing synaptogenesis, stimulating neurogenesis, decreasing neuroinflammation and improving cerebral blood flow (11).Supplementation with Omega 3 PUFA has shown to have improved the oxidative status in the brain tissue homogenate (12).Fish oil is a rich source of EPA and DHA whereas flaxseed oil is rich in ALA. The endeavour of the present study was to investigate the effects of fish oil and flaxseed oil at different pharmacological doses on different cognitive parameters of streptozotocin induced diabetic rats.

MATERIALS AND METHODS

Animals and housing

In house bred male Albino Wistar rats weighing 180–230 g were selected for the study. The rats were maintained under the room temperature and humidity. The rats were fed with standard food pellet and water *ad libitum*. Rats were individually housed in a polypropylene cage with paddy husk as bedding material. Animals were maintained according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experts on Animals (CPCSEA), Government of India.Institutional Animal Ethics Committee (I.A.E.C) approval was obtained before the conduct of the study (IAEC/KMC/2/02/2013) and the rats were handled in humane manner.

Experimental design

Figure 1: Time line of experiment.

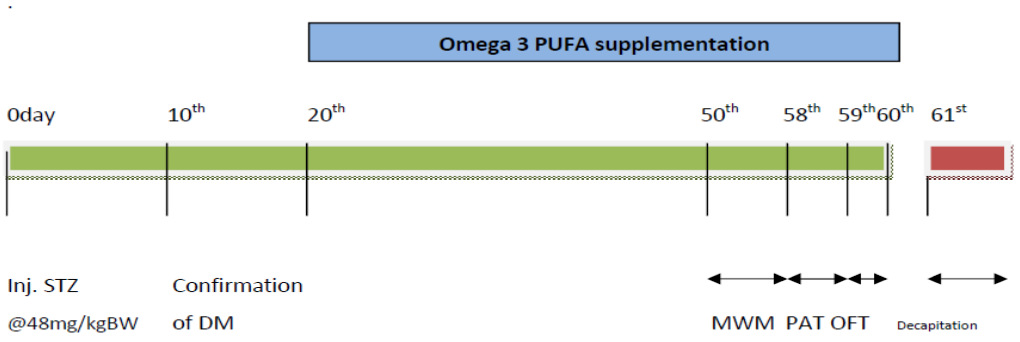

Fig: 1. Experimental time line (not to scale) from Day 0 to 61st. MWM-Morris water maze test; PAT-Passive avoidance test; OFT-Open field test

Animals were randomly divided into six groups.Diabetes was induced by intraperitoneal injection of 48mg/kg body weight of Streptozotocin (STZ) dissolved in citrate buffer at P^H of 4.5.Diabetic state was confirmed by testing the blood glucose level using AccuCheck glucometer on 10^{th} day and on 20^{th} day of STZ injection. Rats having blood glucose level ≥300mg/dL were included in the study. Supplementation was given for 30 days followed by cognitive function test. Animals were sacrificed by decapitation on 60^{th} day. (Fig.1).

Grouping

Rats were divided into following six groups

Group1; n=8: Normal Control rats
Group 2; n=6: Diabetic Control rats (Two animals had died during the study)
Group 3; n=8: Fish oil low dose (FOLD) of 210 mg/kg body weight of omega 3 PUFA (EPA+DHA) which if extrapolated to human dose becomes 2gm/day for a man of 60kg.
Group 4; n=8 Fish oil high dose (FOHD) of 414 mg/kg body weight of omega 3 PUFA (EPA+DHA) which if extrapolated to human dose becomes 4gm/day for a man of 60kg.
Group 5; n=8: Flaxseed oil low dose (Flax LD) of 210 mg/kg body weight of omega 3 PUFA (ALA) which if extrapolated to human dose becomes 2gm/day for a man of 60kg.
Group 6; n=8 Flaxseed oil high dose (Flax HD) of 414 mg/kg body weight of omega 3 PUFA (ALA) which if extrapolated to human dose becomes 4gm/day for a man of 60kg.

Chemicals

Fish oil was procured from Janatha fish meal & oil products, Udupi, Karnataka-India. Flaxseed oil was purchased from a reputed mill in Allahabad. Specification of the fish oil as provided by the Janatha Fish meal & oil products was DHA Min 8% and EPA Min 12%.The oils were orally fed using oral gauge daily for 30 days between 10-11a.m.

Body weight and Random blood sugar levels

Each rat was weighed at the starting of the study and the end of the study. Random blood sugar levels were measured using AccuChek Glucometer at the time of inclusion into the study and at the time of sacrifice.

Behavioural testing was done in all the animals

Conditioned response tests

Morris water maze test (13,14)

It is one of the most widely used methods to access the spatial learning, place learning, cognitive maps and memory in rats. To test the spatial memory, rats were subjected to Morris water maze test from the 30^{th} to 38^{th} of supplementation. The water maze apparatus (Techno, Lucknow, India) consists of a circular water tank of 1.83 m in diameter, painted black and divided into four quadrants by imaginary lines. There was 4"×4" size escape platform submerged in one of the quadrant, the target quadrant. The top surface of the platform was hidden approximately one cm below the surface of the water. The pool was filled with water at a room temperature to a depth of about 40 cm. The rats were trained in the water maze in ten sessions on six consecutive days, with two sessions for four consecutive days except on the first day and last day where only one session was given. Each session consists of four trials. In each trial, time taken to reach the hidden platform was recorded. If the rat was unable to find the platform within one min, the training session was terminated and a maximum score of one min was assigned. Twenty-four hours after the last session, rats were subjected to memory retention. This session was the probe trial and duration was 30 sec (15).Here time taken to reach the target quadrant and the time spent in the target quadrant was noted. Longer the time taken to reach the target quadrant and lesser time spent in the quadrant is an indicative of memory impairment.

Passive avoidance test (16):

Passive avoidance test determines the ability of a rat to remember a foot shock delivered 24 h prior to the memory retention test. The apparatus consists of two compartments: (i) bright, larger compartment and (ii) dark smaller compartment. The smaller compartment was equipped with a grid floor which attached to a foot shock source. Experiment begins by placing a rat in the illuminated larger compartment for exploration. The door between the two compartments remained open at this time. The rat was allowed to explore both the compartments for five min which was followed by three test trials of five min each. At the end of third test trail, as soon as the animal stepped into the dark compartment, the door between the two compartments was closed and a single foot shock was delivered through the grid floor (1.5 mA, 1 sec). The rat was held in the dark compartment for an additional ten sec, to allow the animal to form an association between the properties of the chamber and the foot shock. It was then returned to its home cage. Memory retention test was done 24 h after foot shock. The rat was placed in the bright compartment and the time taken (the step-through latency) for it to enter the dark compartment for the first time was recorded up to 300s cutoff. Longer the latency to enter the dark chamber better is the memory retention.

Unconditioned Response tests

Open Field test (17)

It is one of most widely used methods to access the anxiety-like behaviour, exploratory activities and emotional reactivity in rats. The rats were subjected to open field exploration test on 60th day of the study. The open field apparatus consists of a rectangular box (100×100×60 cm); the floor area is marked into 25 squares (20×20cm).In our experiment we have used a large area open field with more central area as it is better to assess the anxiety, locomotion and exploration behaviour of the rats. A uniform illumination was provided with a 60W bulb fixed above the centre of field. In this novel environment, a rat was placed in one corner of the chamber and its exploratory behaviour for five mins was video recorded. The fraction of total areas crossed in peripheral area (close to wall) and in the central area was noted. In addition to this rearing (elevated hind limb and pelvis with elevation of fore limb) and grooming (use of head, tongue and fore limb for the process of cleaning various part of the body) and defecation scores were quantified.

Statistical Analysis

Statistical analysis was done using SPSS 16th version.Data was expressed as mean ± SEM. Statistical analysis for multiple comparisons was performed by one-way analysis of variance (ANOVA) followed by post hoc test. $p < 0.05$ was considered as statistically significant.

RESULTS

Body weight and Random blood sugar levels

Table 1: Body weight and random blood sugar levels of different groups.

Group	BW-1 (gm)	BW-2 (gm)	p value (Paired t test)	RBS-1 (mg/dL)	RBS-2 (mg/dL)	p value (Paired t test)
Control (n=8)	219.25±5.9	251.75± 6.8	0.001	81.6 ± 4.8	78.7±4.9	0.693
DC (n=6)	210.0±5.2	163.3± 11.4	0.003	449.1 ± 13.75	588.25 ± 6.8	0.00
FOLD (n=8)	204.8 ± 3.7	186 ± 11.9	0.164	558.8 ± 26.2	595 ± 3.2	0.173
FOHD (n=8)	211 ± 5.4	175 ± 6.15	0.000	488.3 ± 30.4	577.3 ± 18.35	0.014
Flax LD (n=8)	217.6 ± 6.6	183 ± 10.1	0.008	427 ± 44.9	542 ± 31.4	0.007
Flax HD (n=8)	208.7 ± 4.7	171.5 ± 11.75	0.005	422.5 ± 34.4	471 ± 40.14	0.007

Data expressed as mean ± SEM

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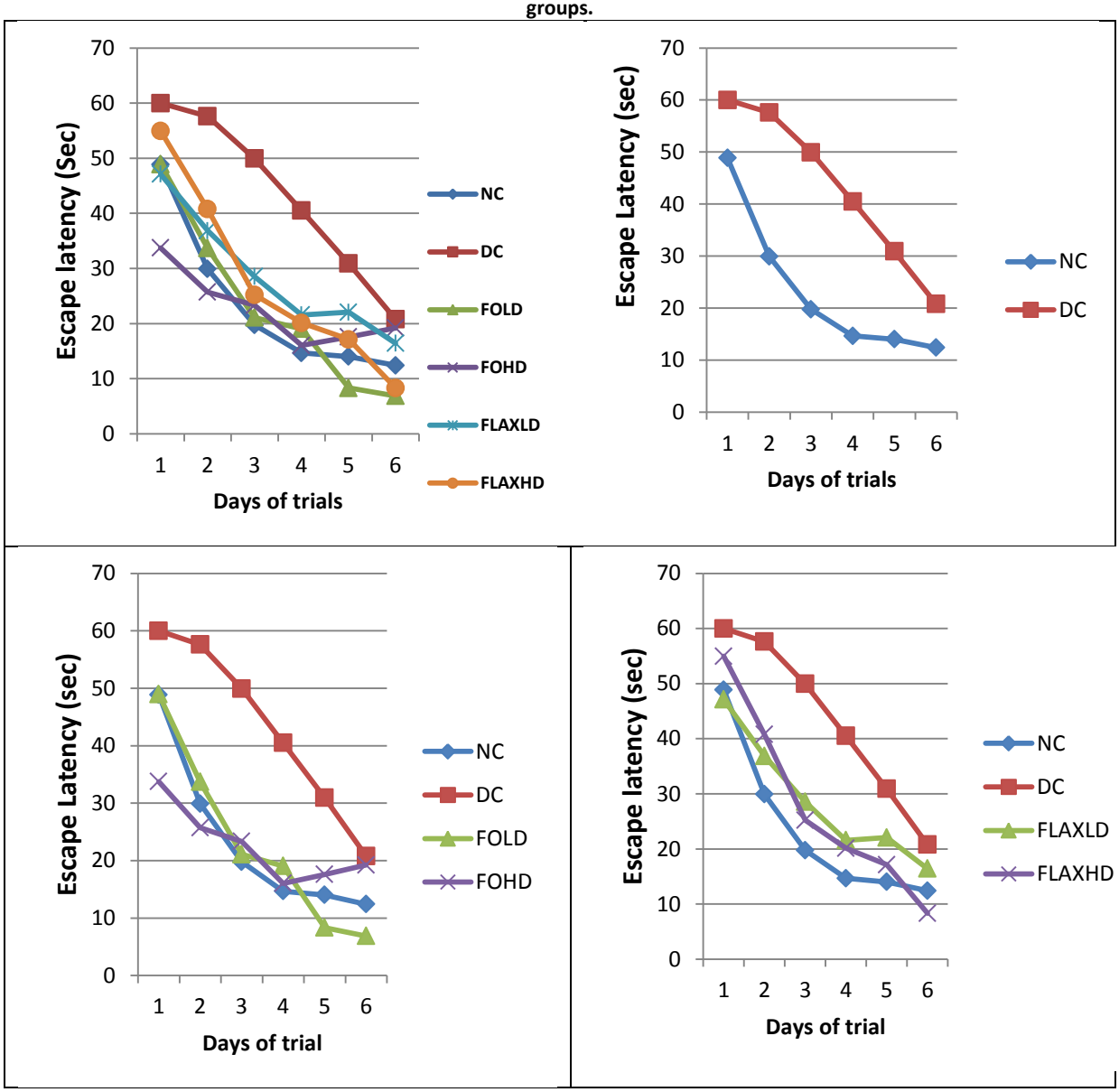
Streptozotocin injection resulted in a diabetic syndrome established by the presence of polydypsia, polyuria, hyperglycemia, and weight loss in the diabetic animals. There was a significant ($p<0.05$) decline in the body weight of all the groups except for the control group and fish oil low dose group from the time of induction into the study and at the end of the study. Hyperglycemic state was maintained throughout the study in all the diabetic rats as shown in Table 1.

Morris Water Maze test: Latency to escape platform during learning session

Figure 2: Latency(sec) to escape the platform during learning sessions of Morris water Maze test by the rats of different groups.



One way ANOVA was done to analyse the learning pattern in the diabetic rats followed by post hoc Tukey's test. Day 1: p=0.001, DC Vs Fish oil high dose (p=0.001).Day 2: p=0.000, Control Vs DC(p=0.000), DC Vs Fish oil low dose (p=0.003), DC Vs Fish oil high dose (p=0.000), DC Vs Flaxseed oil low dose (p=0.012).Day 3: p=0.000, Control Vs DC (p=0.000), DC Vs Fish oil low dose (p=0.000), DC Vs Fish oil high dose (p=0.000), DC Vs Flaxseed oil low dose (p=0.003), DC Vs Flaxseed oil high dose (p=0.000).Day 4: p=0.004, Control Vs DC (p=0.003), DC Vs Fish oil low dose (p=0.019), DC Vs Fish oil high dose (p=0.004), DC Vs Flaxseed oil low dose (p=0.043), DC Vs Flaxseed oil high dose (p=0.024).Day 5: p=0.008, Control Vs DC (p=0.055), DC Vs Fish oil low dose (p=0.004).Day 6: p=0.053.

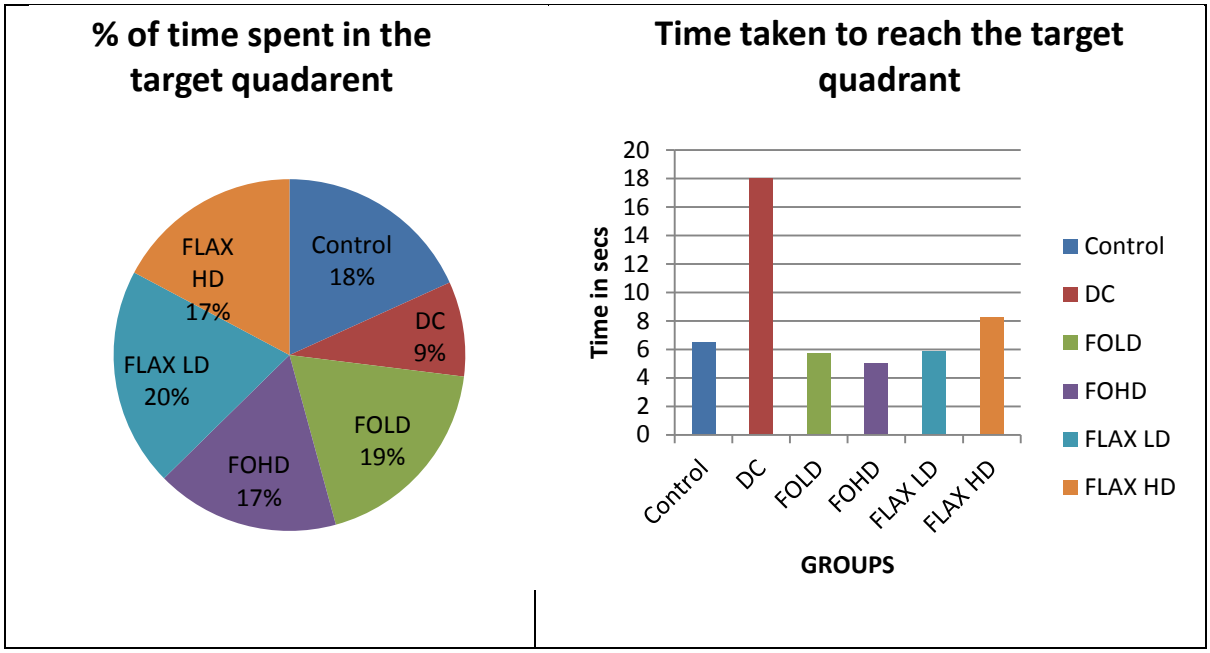
Spatial memory of the rats was examined with the Morris water maze test. On first day rats in all groups went on swimming around the water tank and took longer time to reach the escape platform. The fish

oil high dose treated rats took the least time whereas the diabetic untreated rats took the longest time to reach the platform. On the second day two sessions were done, rats in all groups (except untreated diabetic rats) learned to reach the escape platform quickly and escape there, as their escape latency decreased progressively from session to sessions. Untreated diabetes significantly increased the escape latency, compared with control rats and the omega 3 treated rats ($p<0.05$). These results confirmed that untreated diabetes can induce memory impairment and supplementation with omega 3 PUFA either in the form of fish oil or flaxseed oil improved the learning and memory in these rats. No significant change was observed among the treated groups. (Fig.2)

Time to reach the target quadrant

There was significant effect of omega 3 supplementation on memory retention done during probe trial after 24 hrs of the last session. One Way ANOVA ($F(5, 41) = 15.77$, $p=0.000$) showed a highly significant change in between the different groups. Post hoc test by Tukey's test indicated the mean score for the latency to reach the target quadrant (TTP) by fish oil and flaxseed oil treated groups of both the doses was significantly($p<0.05$) lesser than the diabetic control (Fig.3).

Figure 3: Probe trial results of Morris Water Maze showing the time taken to reach the target quadrant and the time spent in the target quadrant. Diabetic rats took longest time to reach the platform, while Fish oil (high dose) treated animals showed least time.



Percentage of time spent in the target quadrant

One Way ANOVA test showed a significant increase ($F(5, 41) = 4.32$, $p=0.003$) in the percentage of time spent by the omega 3 PUFA treated rats as compared to the untreated diabetic rats. Post hoc Tukey's test showed a significant ($p<0.05$) increase in the time spent by all the groups of omega 3 treated rats as compared to the untreated diabetic rats. (Fig.3)

Passive Avoidance Test

One Way ANOVA was done for both acquisition trial and retention trial. There was no statistically significant change in acquisition trial ($p=.174$) where as there was statistically significant change is retention trial done on the second day ($p=0.028$). In retention trial Post hoc test by Tukey's HSD showed significant ($p<0.05$) change in between DC & Fish oil low dose , DC & Fish oil high dose ,DC& Flax LD.(Fig.4)

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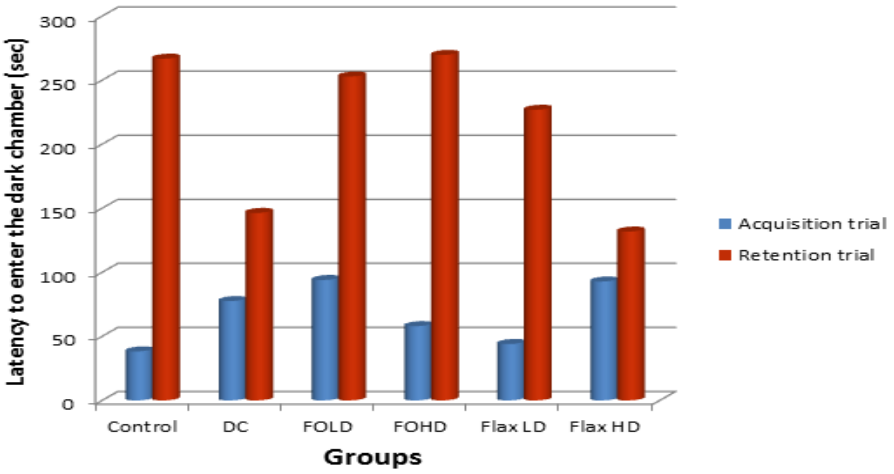
Figure 4: Passive avoidance test results of the different groups. Retention of memory was least in diabetic rats and highest in Fish oil treated animals.



Open field test

One way ANOVA test showed a significant (F (5, 40) =3.2, p=0.015) change among the groups in crossing the number of central squares. There was no significant change in peripheral squares crossed. Post hoc test showed a significant change ($p<0.05$) in number of central squares crossed in between the following groups, Control Vs DC, Control Vs FOLD, Control Vs FOHD, Control Vs FlaxLD.Table.2

One way ANOVA test showed no significant change in rearing, grooming and defecation scores in the different groups.Table.3

Table 2: Open Field Test results

Group	Peripheral Squares Crossed	Central Squares Crossed
Control (8)	31.25 ± 5.5	5.3 ± 2.08
D C (6)	16.5 ±7.6	0.5 ± 0.34*
FOLD (8)	11.1 ±2.5	1 ± 0.65*
FOHD (8)	20.5 ±4.7	0.62 ± 0.49*
Flax LD (8)	15.5 ± 3.8	0.62 ± 0.49*
Flax HD (8)	19 ± 8.09	1.8 ± 0.87

*=p<0.05, Data expressed as mean ± SEM

Table 3: Open Field Test results showing ambulation scores.

Group	Rearing Scores	Grooming Scores	Defecation Scores
Control (8)	11 ± 1.03	5.5 ± 1.8	2.8 ± 0.5
DC (6)	3.8 ± 1.8	3 ± 1.2	2.6 ± 1.08
FOLD (8)	6.6 ± 1.74	3.7 ± 1.06	3.2 ± 0.59
FOHD (8)	7.8 ± 1.44	5.2 ± 0.25	2.3 ± 0.77
Flax LD (8)	6.2 ± 1.12	5.37 ± 1.03	4.2 ± 0.79
Flax HD (8)	8.5 ± 2.18	5 ± 1.32	3.3 ± 0.62

Data expressed as mean ± SEM

DISCUSSION

There was a decline in the body weight of the rats recruited for the study except for the control rats where there was an increase. Diabetic state was maintained through out the study and is evident from the random blood sugar levels recorded at the end of the study. Omega 3 PUFA supplementation given both in the form of fish oil and flaxseed oil had no hypoglycaemic effect in our study, results of which are in agreement with other studies (10,18).

Affirmation of cognitive dysfunction in untreated diabetes is done by the results of Morris water Maze test, Passive avoidance test and Open field test where the untreated diabetic rat's performances was significantly poor as compared to the control group rats. The results are in agreement with the previous studies(19–22).The present study examined the effect of dietary supplementation omega 3 PUFA both in the form of fish oil and flaxseed oil at different pharmacological doses on cognitive dysfunctions of diabetic rats. Both fish oil and flaxseed oils showed beneficial effects on the parameters of cognitive tests.

Spatial memory tested by Morris Water Maze test is strongly dependent on hippocampal activity and requires neural activity in the CA1 sub-region of the hippocampus (23). Supplementation with Omega 3 PUFA in diabetic rats conducts Neuro protective function through an anti-apoptotic pathway and significantly improves the ability of learning and memory (10).The water maze training increases the level of neurotransmitter in the hippocampal CA1 region (24).Several studies have reported that diabetes caused apoptosis-induced neuronal loss in hippocampus and to a lesser extent in the frontal cortex of rats which associated with cognitive impairment (25.26).In our study omega 3 supplemented groups exhibited significant ($p<0.05$) reduction in their escape latencies to find the platform as compared to the untreated diabetic groups during Morris water maze spatial learning sessions of Day two to Day five. (Fig.2) In probe trial which is administered after 24hrs all the treated group rats reached the platform quadrant in less time and had spent significantly more time in the platform quadrant as compared to the untreated diabetic rats (Fig.3).Our study confirms the improvement in cognitive function similar to the previous study (27), we further have shown the similar beneficial effect in flaxseed oil treated groups also. Here the omega 3 through its multifaceted action as an anti-inflammatory, anti-oxidative and anti-apoptotic(10) would improve the learning and memory in diabetic rats.

Passive avoidance test assessing the associative memory (28) showed the beneficial effect of omega3 supplementation on diabetic rats during retention trial done after 24 hrs of shock. In treated groups there is significant decrease in the latency to enter the dark compartment in the treated groups as compared to the untreated animal indicating better memory retention. (Fig.4)

The reported observations of locomotion and anxiety behaviour tested by open field test revealed that diabetic animals show increased anxiety and depression as compared to non diabetic (22). In our study omega 3 PUFA supplemented animals exhibited increased activity during the open field task suggesting a reduction of their level of anxiety. As reported by a study (29)anxiolytic treatments by omega 3 PUFA (as in our case) do not themselves increase exploration in the open field but decrease the stress-induced inhibition of exploration behaviour. However we did observe significant changes in the number of crossings of the central zone, a parameter closely linked to anxiety, suggesting a moderate anxiolytic effect of long chain omega 3 PUFA, which is in concomitant with the previous studies (30, 31).There was no significant difference in the cognitive parameters in between the different treated group of rats.

From this study we suggest that both source of omega 3 PUFA administered for 30 days to diabetic rat models showed distinct improvement. Therefore we firmly believe that these food supplements will greatly benefit millions of patients suffering from DM. As both the source of omega 3 PUFA is used in the study, this could be a great benefit for the patients of all types of food habits.

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