DISCUSSION

Neuroprotective effect of omega-3 PUFA rich fish oil and flaxseed oil along with the vitamin C and vitamin B\textsubscript{12} supplementation was analyzed by various parameters such as behavior of the animals in different mazes used for testing cognitive parameters, brain oxidative status, neuroinflammation and hippocampal BDNF levels on STZ induced diabetic rats. We preferred STZ induced diabetic models for our study because it acts as an excellent model to study the cellular, molecular and morphological changes in the diabetic brain and provides a pertinent example of endogenous chronic oxidative stress because of hyperglycemia. (423,424) Cognitive parameters mainly focused on the learning and memory aspect of cognition which was supported with the biochemical findings in the different parts of the brain involved in the storage and processing of information regarding learning and memory. (425) In the present study, we emphasized on the biochemical changes in the frontal cortex, hippocampus and cerebellum and stressed on their correlation with the cognitive functions in the diabetic rat models.

7.1. Effect of omega-3 PUFA rich oils and vitamin C and vitamin B\textsubscript{12} on Body weight, Random blood glucose level, Brain weight

Albino male Wistar rats of 2-3 months with body weight in the range of 200-250 grams were selected and recruited in the study. They were maintained in the standard housing conditions and following a preliminary evaluation, experimental group of rats were induced with diabetes. Normal control and diabetic control group was kept and maintained parallel to the treated groups. Severe diabetes type 1 was induced by injecting a single dose of streptozotocin at 48 mg/kg body weight intraperitoneally. By the third day of injection the animals presented the features of diabetes, but in our study a waiting period of 10 days was observed for the confirmation of diabetes as it was observed that some animals reverted to the normoglycemic state. Following a wait period of 10 days, rats with blood glucose levels (> 300 mg/dL) were recruited and subdivided into different groups. A lag period of 20 days was observed for the development of features of diabetes mellitus with long duration.
After 20 days, dietary supplementation with omega-3 rich fish oil and flaxseed oil in two different doses, i.e. low dose and high dose only and in combination with vitamin C and vitamin B_{12} was administered. Male Wistar rats of the experimental groups were treated with the dietary supplement for a period of one month except for the control and diabetic control group of animals. A constant hyperglycemic state was observed in the animals throughout the study period. Animals recruited for the study continued to appear ill-looking and had gradual and continuous loss of their body weight because of the adverse effects of STZ. STZ due to its DNA alkylation and damage produced hyperglycemia in these rats. Loss of structural proteins, the major contributor to body weight, led to reduction in body weight from the day of recruitment to the day of sacrifice. A significant reduction in body weight and a sustained hyperglycemia indicated successful induction of diabetes and the animals mimicked the features of untreated diabetic patients. 

Some of studies have shown the antidiabetic action of omega-3 PUFA. Iwase et al. 2015 have discussed the two different pathways of antidiabetic role of omega-3 PUFA, one via secretion of GLP-1 mediated by GPR120 and the other mediated by SREBP and PPAR. But in our present study we found no hypoglycemic effect of omega-3 PUFA and vitamin supplementation, and this corroborates with results from other studies. Unlike other studies, we did not find any beneficial synergistic effect of vitamin supplementation on the blood glucose and lipid profile of the diabetic rats either.

Weights of the different parts of the brain such as cerebral cortex, frontal cortex, hippocampus and cerebellum were noted at the time of sacrifice. To reduce the bias and to facilitate comparisons in between the different experimental groups relative weight of these brain parts were calculated. There was no significant change in the absolute weight of the cerebral cortex but a significant change in the absolute weight of cerebellum, frontal cortex and hippocampus was observed across the study groups. A significant change was also observed in the relative weights of cerebral cortex, cerebellum, frontal cortex, and hippocampus of the animals. Relative brain weights were more in the STZ induced diabetic rats due to a decrease in their body weight. Although diabetes had caused a huge loss of body muscle mass but it had
minimal effect on the brain tissue weight as evident from the results of this study. (Table 6, 7, 7.1)

We tried assessing the biochemical changes specially the oxidative status, neuroinflammation and neurotrophic levels not only in the hippocampus but also in the frontal cortex and cerebellum. Apart from its classical role in fine motor control, increasing evidences have proved that cerebellum has variety of other non-motor functions such as attention, sensory discrimination and learning and memory. (433) Cerebellum is essential for the storage of procedural memories. Moreover, the cerebellar cortex is susceptible to hyperglycemia induced oxidative stress and this could further contribute to the neuronal damage (424) leading to cognitive decline in diabetic rats. Along with hippocampus the prefrontal cortex also is involved in storage of short term memories and play vital role in cognition. Thus, through this study an attempt was made to consider the biochemical and physical changes in these areas of brain in diabetic rats.

7.2. Effect of omega-3 PUFA rich oils and vitamin C and vitamin B_{12} supplementation on diabetes induced cognitive dysfunction

Experimental group of animals were subjected to evaluation of cognitive functions by the different maze tests namely; Morris water maze test, Passive avoidance memory retention test, Open field test and Elevated maze plus test. In our study rodents with diabetes showed impaired results in cognitive tasks such as increased latency during training and probe trial sessions of MWM test, enhanced retention for passive avoidance training and disruptions of open field activity which are similar to the results from other studies. (434–436) Pathogenesis of learning and memory impairment in diabetic condition is multifactorial and has not been explained explicitly. Diabetes specific risk factors (e.g. hyperglycemia, insulin deficiency, and insulin resistance), diabetes linked vascular factors (e.g. hypertension, hyperlipidemia, and obesity), microvascular complications (e.g. stroke), neuronal alterations, degeneration; neuronal loss might be collectively associated with cognitive dysfunction in diabetes. (143,437,438) Some of underlying mechanisms have been discussed in the review section. Deleterious effects of diabetes on brain structure and various domains of cognition have been reported in both experimental and clinical
It is now well known that diabetes in rodents can develop cognitive decline like those observed in diabetic patients mainly with relation to learning and memory. Chronic hyperglycemia leads to several metabolic changes as well as changes in enzymatic activities considered essential for central nervous system function, such as aminolevulinate dehydratase (d-ALA-D), Na+, K+-ATPase, and acetylcholinesterase (AChE). Diabetes mellitus leads to decreased Na+, K+-ATPase activity which could be linked directly to cognitive changes in diabetics. Deficits in learning and memory are also related to the increased acetylcholinesterase activity which in turn is associated with increased polymerization of β-amyloid plaque responsible for cognitive deficient in Alzheimer disease.

Hippocampal damage in the diabetic animals leads to profound deficits in spatial memory, thus indicating the role of hippocampus in mapping of space. Recent evidences are supporting the fact that memory and spatial functions of hippocampus can be reconciled. Electrophysiological studies on hippocampus of STZ rats have revealed an increase in epileptiform activity in CA3 region, reduced expression of long term potentiation and increased hypersensitivity to ischemic insults of CA1 area and neuronal necrosis in CA2 and CA3 sub regions. All these changes are associated with impaired water maze learning in STZ induced diabetic rats.

In the present study, the untreated diabetic control rats failed to learn and memorize the escape pathways and their escape latencies were significantly increased. Results from the probe trial test, done 24h after the last training session also established the adverse effect of diabetes on spatial memory. The diabetic control group of rats showed longer latency to reach the target quadrant and spent lesser time in the target quadrant in comparison to the control group. These results agree with results from other studies. Rats of the treated groups showed a significant shorter latency to escape on the platform as compared to the untreated group of rats. Groups with higher dose of fish oil or flaxseed oil administered alone or in combination of vitamin C and vitamin B12 took shorter time as compared to the parallel group of lower dose of fish oil and flaxseed oil. No significant difference in the latency during training sessions was observed in between...
the fish oil treated groups and flaxseed oil treated groups. Probe trial results also reflected the similar results as of the learning sessions of MWM. Findings of other studies (143,320) have shown the beneficial effect of fish oil on learning and memory as assessed by MWM test in the rodents. Our results also support and augment these findings by showing a similar result not only in groups treated with animal source of omega-3 (fish oil treated groups) but also in groups treated with plant source of omega-3 (flaxseed oil supplemented groups). (Table 8 & 9 Figure 10, 11, & 11.1)

For passive avoidance memory retention test, memory retention ability was affected by untreated diabetes in diabetic control group of animals. The results of the present study are consistent with earlier reports. (436,440,447) Untreated diabetic rats had poor memory retention and they entered to the dark chamber where they were given foot shock on the second day of passive avoidance test. Whereas the treated group of animals had better memory retention and took longer time or did not enter (Fish oil high dose & flaxseed high dose + vitamin C group) the dark chamber at all. Untreated diabetic control rats spent lesser time in the bright chamber of passive avoidance apparatus, unlike the rats supplemented with omega-3 rich oils and vitamin C and B12. (Table 10, Figure 12, & 12.1)

Omega-3 PUFA supplementation increases the brain DHA content in the phospholipid membranes of excitatory neurons, thus affecting the neuronal conduction and transmission. Dietary supplementation decreases the neuronal arachidonic acid, inhibits phospholipase A2 and restores the LTP expression in the hippocampus. (448,449) As optimal level of unsaturated fatty acids is necessary for the maintenance of neuronal membrane structure and fluidity, a marked scarcity of these fatty acids adversely affects the functions of the membrane bound enzymes, ion channels, and receptors. Activities of enzymes related to signal transduction such as Na⁺, K⁺-ATPase and protein kinase C are also adversely affected due to the n-3 PUFA deficiency. Omega-3 PUFA supplementation stabilizes the neuronal membrane and in turn improves the synaptosomal Na⁺, K⁺-ATPase activity and facilitates the NMDA responses. (450–452) Omega-3 PUFA modulates the expression of the genes, cytoskeleton of the membrane, synaptic plasticity, signal transduction, hippocampal nerve growth, ion channel formation, energy metabolism, and regulatory proteins. (453–455) It also improves the brain mitochondrial functions and enhances the levels
of anti-apoptotic Bcl-2 and neurotrophic neuroprotectin D-1 (NPD-1) like metabolites (456) emphasizing on its neuroprotective role in aged brain, features of which are like the diabetic brain. (26) Changes diabetic brain resembles the aged brain changes. It has been observed that supplementation with omega-3 PUFA in aged rats increases the cerebral choline and acetylcholine levels as evident from their better performance in passive avoidance test and maze learning ability. (457,458) In a recent study it was observed that supplementation of DHA improved the impaired learning and memory by balancing the glutamate and GABA levels in tissue samples of hippocampus. (459) Glutamate is an excitatory neurotransmitter whereas GABA is an inhibitory neurotransmitter and a balance of these neurotransmitters is essential for the proper functioning of brain such as in learning and memory process, locomotor activity and circadian rhythms. (460) Pre-administration of DHA in AD rat models prevents the deposition of beta amyloid in the hippocampus and the parietal cortex by inhibiting α- and β-secretase activity, thus reduces the impairment of learning ability. (461,462) Owing to these multidimensional effects, we found a positive influence of omega-3 PUFA supplementation on learning and memory behavior in diabetic rats.

In the present study, we observed that diabetes significantly reduced the locomotion among rats, as evidenced by the decreased number of peripheral squares moved by the untreated rats and reduced ambulation in the central square area of open field apparatus. Treated rats showed an increased ambulation with the highest being in fish oil and flaxseed oil with vitamin B_{12} group of rats. Similar results are reflected in the percentage of time spent in peripheral and central squares. ([Table 11, Figure 13, 13.1, 14 & 14.1](#)). Increased movement or decreased latency to enter the central area is indications of anxiolysis. (463) In open field apparatus anxiety behavior of rats are initiated by two factors: individual testing (as the animal is isolated from its social group) and agoraphobia (due to the larger space of the apparatus in comparison to the animal’s normal habitat of the cage).

Hence in this experiment we have tested the effect of treatment on the reaction of the rodents to a stressful situation. Likewise, elevated maze plus results also showed that the STZ diabetic rats spent significantly more time in the closed arm than in open arms, there immobilization or freezing time was increased with less number of rearing and grooming in the closed arms. Dietary supplementation with fish oil and flaxseed
Description

Fish oil along with vitamin C and B12 showed a significant increase in the entries to the open arms of the elevated plus maze apparatus. Fish oil high dose group had the highest number of entries to the open arms in comparison to the rest of the groups. The percentage of time spent in the arms also reflected a similar result as the number of entries to the arms. STZ induced diabetic rats spent lesser time in the open arms in comparison to the normal control rats. The percentage of time spent by the animals in the open arms also increased in the treated group with the highest being in flaxseed oil high dose group and lowest in flaxseed oil low dose and vitamin C and B12 treated groups. The percentage of time spent in closed arm is the reverse of open arm time, with STZ rats spending the maximum time in the closed arms. A significant effect of dietary supplementation on the rearing and grooming scores in the closed arms of elevated plus maze was observed. Immobilization time was more in the diabetic rats, which gets reduced with the supplementation of omega-3 PUFA and vitamins. (Table 12, 13 & Figure 15, 15.1, 16, 16.1, 17 & 18)

All these behavioral responses are indicative of anxiogenic effect of diabetes and the results of the present study agree with other studies. (464–467) Evidences from the literature suggest that T1DM is strongly associated with the risk of affective and anxiety disorders (468–470). STZ induced diabetic rats showed increased anxiety-like behavior in different behavioral paradigms such as Elevated plus maze (EPM), Open field test (OFT) and Zero maze (465). The precise mechanism underlying the association between diabetes and anxiety is not clearly known. There are some suggestions regarding alterations in the neurotransmitter (GABA and monoaminergic) and neuroendocrine (HPA-axis) systems that might be associated with anxiety in diabetes. (466,471,472) The homeostasis of these neurotransmitter system in the brain is disrupted by diabetes, leading to the development of psychological complications. Modulation of GABAergic system is involved in the pathophysiology of anxiety (473) and forms the basis of action of several anxiolytic drugs in clinical use such as diazepam, and other benzodiazepines. (474) Diabetes also alters the brain monoaminergic functions which underlie pathophysiology of anxiolytic behavior in these subjects.

Amygdala plays an important role in modulating anxiety as sensory information of the environment is conveyed to lateral and basolateral amygdaloid (BLA) nuclei where it
gets processed locally and then is sent to the central nucleus (CeA) where an appropriate anxiety response is executed. (475) Dopaminergic neurotransmission has a major effect on the amygdaloidal modulation of anxiety. Studies have proved that diabetes influences the dopaminergic neurotransmitter system within the different brain regions such as caudate, putamen, medial and lateral pallidus, medial hypothalamus, locus ceruleus, ventral tegmental area, amygdala and hippocampus. Blocking of amygdaloid Dopamine D1 receptor prevents the anxiety behavior of the rats. (464) Omega-3 PUFA supplementation modulates the dopaminergic neurotransmission by elevating the binding of dopamine neurotransmitter to the D2 receptors and increasing the endogenous dopamine levels in the frontal cortex and nucleus accumbens (334,476) which plays important role in cognitive and affective functioning. (477) It also checks the hyperactivation of HPA axis, thereby decreasing the corticosterone effect on dendritic morphology and neuronal activity in the basolateral amygdala and bed nucleus of the stria terminalis (BNST), major centers regulating anxiety. Omega-3 PUFA increases the serotonin levels in the brain which reduces the neuronal excitability. (478)

In the present work, we observed an enhancement in the learning and memory in the diabetic rats on supplementation of omega-3 rich oils and vitamins as reflected by the probe trial results.

Omega-3 PUFA supplementation reduced the anxiety in the diabetic rats as reflected by the number of entries and time percentage spent in the open arm. Increase in exploration time in the open field maze was observed in treated group, the findings are in cohort with other study on restraint stress. (479) Thus, omega-3 PUFA has anxiolytic effect on STZ induced diabetic rats.

There was no significant difference in the cognitive function test results of omega-3 rich oils supplemented groups and vitamin C and B12 supplemented combination groups. These findings preclude from drawing an inference about the specific role of vitamins given alone or in combination on different cognitive parameters of diabetic rats. Hence, the observed improvement in the cognitive functions might be attributed majorly to the omega-3 rich fish oil and flaxseed oil. We also observed that there was no significant difference in the cognitive parameters of fish oil treated groups and flaxseed oil treated groups, suggesting that plant source
omega-3 PUFA have equal and similar beneficial effects as that of omega-3 PUFA from animal sources.

7.3. Effect of omega-3 PUFA rich oils and Vitamin C and B₁₂ on lipid profile and brain oxidative status

Diabetic condition manifests with hypertriglyceridemia, hypercholesterolemia and lower levels of high-density lipoprotein cholesterol (HDL). In the current study, untreated diabetic rats had high TG and TC levels and low HDL levels as compared to the controls. (Table 14 & Figure 19) Supplementation of omega-3 rich fish oil and flaxseed oil had significant TG, TC lowering effect and HDL increasing effect, most effective being in the fish oil high dose (FOHD) group. The outcomes of the present study are in line with the other studies. (232,429,480,481) These altered lipid profile in diabetes are not only correlated with increased risk of cardiovascular diseases and cerebrovascular diseases, but are also equally associated with risk factors for dementia and cognitive impairments. (482,483)

The underlying mechanisms of omega-3 lowering effect on TG levels supplemented at pharmacological dose are: (a) reduced hepatic lipogenesis due to decreased enzymatic conversion of acetyl CoA to fatty acids; (b) increased beta oxidation of fatty acids; and (c) activation of transcription factors controlling metabolic pathways regulating nutrient traffic and reducing plasma TG. (232,484)

Vitamin B₁₂ acts as a coenzyme of methylmalonyl CoA (MM-CoA) to succinyl-CoA. Deficiency of vitamin B₁₂ results in the accumulation of MM-CoA that inhibits the rate limiting enzyme, carnitine palmitoyl transferase (CPT1) of fatty acid oxidation. Thus, regulating the enzyme acetyl CoA carboxylase (ACC) activity (485) causes lipogenesis. This might be the linking mechanism between vitamin B₁₂ deficiency and lipid disorder seen in the diabetic conditions. Finding from an Indian study also showed an independent association of vitamin B₁₂ with TG and HDL in diabetic patients. (486) But in our experimental study on diabetic rat model we found no significant difference in the lipid profile of the vitamin B₁₂ treated group of animals, which is in concordance with results of other study on T2DM. (487) (Table 14 & Figure 19)
Although in the present study a synergistic effect of vitamin B\textsubscript{12} and omega-3 rich fish oil and flaxseed oil lowered the TG and TC levels and increased HDL levels like the results from other studies, (488,489) there was no difference in between the omega-3 rich oils supplemented only and vitamin B\textsubscript{12} combination groups. We observed a negative correlation between TG and memory performance as assessed by the probe trial parameter i.e. time spent by the animals in the target quadrant. (Table 9 & Figure 19.1 &19.2) Linear regression test indicated TG to be a better predictor for the time spent in the target quadrant. Thus, we can also state that elevated TG levels in diabetes could be another enhancing factor for cognitive dysfunction.

To the best of our knowledge, the present study is the only showing a relationship between TG, TC, HDL and memory in an experimental study of STZ induced diabetic rats treated with two different source of omega-3 PUFA in two different doses. Role of dyslipidemia and their effects on cognition remains unclear and inconsistent. While some studies have stressed on the association of dyslipidemia with dementia, (490–492) others have reported no relation between dyslipidemia and cognitive impairment. (493–495) In a community based study it was observed that high cholesterol, higher HDL-C and lower TG were not significantly associated with better cognition once the confounding effects of childhood IQ was considered. (496) As it is evident from the results of the current study and other published studies, diabetes of both the types are known to have an association with poorer cognitive performances. Results of studies on specific impact of each of the component of lipid profile on cognition have been very vague. Some have observed a positive association between the higher levels of LDL (low density lipoprotein) cholesterol and total cholesterol with memory in late life (greater than 85 years of age) individuals without APOE4 allele, (497) while another study on aged population found a relationship between low HDL and cognitive impairment and no association with low LDL and TG (498). Association of elevated triglycerides with poorer executive function has been discussed in a recent study on bipolar disorder. (499) The possible mechanisms postulated are: - a) hypertriglyceridemia along with other components of metabolic syndrome such as obesity, hypertension, low HDL and hyperglycemia may damage the cerebral microvasculature thus leading to cognitive impairment (500); and b)
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elevated TG levels may prevent the entry of leptin hormones into the brain via blood brain barrier thus, affecting the cognition, for leptin once in brain enhances cognition. (501,502) Some of the in vitro studies have also shown the effect of cholesterol (503) and HDL cholesterol (504) on the formation of amyloid-β, main constituent of amyloid plaques. In many of the cross-sectional studies (498,505) high density cholesterol has been suggested to be protective against memory impairment and some have shown to be associated with better cognitive function. (506)

In the present study, there was a significant correlation between HDL and memory retention as assessed by the probe trial, increase in HDL level is negatively correlated with PT-1 (time the animal takes to reach the target quadrant) and positively with the PT-2 (time spent by the animal in the target quadrant). These results indicate better memory retention in the rats with high HDL. (Table 14.1, 4.2 & Figure 19.2, 19.3). Treatment with omega-3 PUFA and vitamins improved the HDL levels and decreased the TG and TC levels in the diabetic rats and enhanced the cognition in diabetic rats as seen in the behavioral test performances. Hence, we can conclude that in the present study there is an association between dyslipidemia and memory impairment, which on treatment with omega-3 rich oils and vitamin and B₁₂ gets ameliorated.

In diabetic brain, auto oxidation of glucose, lipid peroxidation, decreased tissue concentration of reduced glutathione, impaired activities of antioxidant defense enzymes such as catalase and superoxide dismutase (SOD) are the possible sources for oxidative stress. (65,507,508) A high metabolic rate in the neurons leads to a higher baseline production of ROS. Because of its structural and biochemical properties such as high levels of peroxidable fatty acids, elevated consumption of O₂, scarcity of antioxidant systems, and abundance in iron content, brain is particularly sensitive to oxidative stress. (509,510) Reactive oxygen species act as messenger molecules in long term potentiation. (511) Moderate levels of ROS are considered to enhance the peripheral insulin sensitivity via oxidative modification of the insulin receptor. (512) However, an imbalance in ROS production from the mitochondria and the antioxidant defense system leads to oxidative stress related to peripheral insulin resistance as well as pathophysiology of AD. (512,513) Oxidative damage to the protein and lipids of neuronal cell membrane disturbs the physiological activities of the membrane bound enzymes, ion channels and receptors. Lipid peroxides,
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carbonyls, advanced glycation end products (AGEs) and advanced oxidation protein products (AOPPs) attack other molecules generating free radicals which results in protein unfolding, inactivation and aggregation. All the above changes alter the normal functions of the neurons leading cognitive decline in diabetes.

In the current study, we observed a significant increase in the lipid peroxidation in the hippocampus, cerebellum and frontal cortex of the STZ rats as compared to the controls. (Table 15 and Figure 20)

Hippocampus plays an important role in spatial memory and due to its vulnerability to oxidative stress, it is one of the most affected part of the brain. It is evident from our results, where we found the hippocampal MDA levels of the diabetic rats to be higher as compared to the MDA levels of frontal cortex and cerebellum. The findings are similar to the other study done by Cardoso, et al. (515) Unlike other studies, we did not find any correlation between MDA values from hippocampus, cerebellum and frontal cortex with the probe trial findings of Morris water maze test, a marker for memory testing. (Table 15.1)

There was a significant increase in the protein carbonyl content of the diabetic control rats in comparison to the controls indicating an increased protein oxidation in the STZ induced diabetic rats. (Table 15 and Figure 21)

We also found a positive correlation between the protein carbonyl content and PT-1 (time taken by the animal to reach the target quadrant) during the probe trial of the MWM test, an indicator for memory assessment. Higher the value, longer was the time taken by the rats to reach the target quadrant, indicating impairment of memory in the STZ induced diabetic rats (Table 15.1 and Figure 21.1).

Carbonylation leads to irreversible modification of proteins causing their structural and functional alterations leading to cellular dysfunction and tissue damage. (516) Diverse functional proteins involved in nucleocytoplasmic transport, immunity and defense, ubiquitination-proteasome pathway, energy metabolism, neurotransmitter and purine metabolism are vulnerable to oxidative damage. (517) Aggregation of cross linked proteins leads to further oxidative damage and finally may lead to apoptosis of the neuronal cells. Increased accumulation of carbonyl proteins in the brain has been regarded as the causative agent for the age-related neurodegeneration. (518) In Alzheimer’s disease there is high accumulation of carbonylated proteins
which are found to be localized to the paired helical filaments (PHF) and amyloid plaques, suggesting their crucial function in the advancement of the neurodegenerative disease. (519)

Treatment with omega-3 rich fish oil and flaxseed oil discretely and in combination with vitamin C and B\textsubscript{12} showed a beneficial effect on the MDA and protein carbonyl levels. There was a reduction in the MDA levels in all the three parts of brain, lowest being in the fish oil high dose group of animals (Table 15). These results are in agreement with previous findings on aged Wistar rats (520) and in blood, liver and heart homogenates of STZ induced diabetic rats. (521) Dietary supplementation showed the protein carbonyl lowering effect in the STZ induced diabetic rats. Low dose of flaxseed oil in combination with vitamin C and vitamin B\textsubscript{12} showed the best result with lowest value of plasma protein carbonyl (Table 15). Although the exact molecular mechanism involved in the antioxidant action of omega-3 PUFA cannot be fully explained but certain theories have been postulated such as inhibition of NADPH oxidase isoform Nox 4 expression and activity in the vascular endothelial cells. It is further postulated that group V sPLA\textsubscript{2} (secreted phospholipases A\textsubscript{2}) downregulates Nox 4 with the involvement of extracellular signal-related kinase (ERK) and protein kinase C (PKC), thus reducing the ROS production in cardiovascular diseases. (522) which might be one of the mechanisms for the reduction of oxidative stress in the brain regions of diabetic rats.

Recent studies have revealed that EPA derived E-series resolvins (RvE1 and RvE2) and DHA derived D-series resolvins (RvD1 and RvD2) and neuroprotectins have potent antioxidant and anti-inflammatory properties. (523) Study by Qiang Liu, 2014 reported the protective effect of DHA against the oxidative injury to the neural progenitor cells (NPC) via Nrf-ARE pathway (524) which suggests the antioxidative property of omega-3 PUFA and their beneficial role in neurodegeneration. Several experimental and clinical studies have proved the fact that increase in oxidative stress and reduction in antioxidant levels are the core causes for the pathophysiology of cognitive decline in diabetes mellitus.

Administration of antioxidants for the treatment of various diseases have given contradictory results, as it might be that many of the studies had focussed in the use of antioxidant as a blocker of ROS production and other compounds rather than
focussing on blocking the changes in intracellular signalling produced by oxidative stress. (507)

Omega-3 PUFA especially DHA increases the levels of antioxidant enzymes such as glutathione peroxidase, catalase, reduced glutathione levels in the cortex and hippocampus of diabetes rat models. (525) Glutathione has an important role in cell proliferation, regulation of apoptosis, as an enzyme cofactor, a cysteine storage form, a major redox buffer, and a defence against variety of different ROS. (526,527) Disturbance in the glutathione homeostasis have been associated with pathogenesis of many neurodegenerative diseases.

In the present study, we observed an increase in glutathione levels in different brain areas of treated group of rats. In high dose fish oil and vitamin B_{12} group hippocampal GSH level was close to that of control group animals. The fish oil high dose administered group had the highest frontal cortex GSH level whereas the low dose flaxseed oil in combination with vitamin C group had the highest cerebellar GSH level as compared to the other treated groups. These findings are indicative of beneficial effect of the dietary supplementation on the antioxidant enzyme levels in the brain region.

A significant negative correlation between frontal cortex GSH level with the PT-1 (time taken by the animal to reach the target quadrant) during probe trial of MWM was observed. Higher was the value of GSH in the frontal cortex shorter was the time taken by the animal to reach the quadrant, indicating a better memory. Rats with higher frontal cortex GSH levels spent longer time in the target quadrant as implied by the significant positive correlation of GSH frontal cortex and PT-2 (time spent by the animal in the target quadrant) during the probe trial of MWM test. (Table 17.1 & Figure 24).

A significant decrease in total antioxidant status as assessed by FRAP levels in all the three parts of the brain (i.e. hippocampus, frontal cortex and cerebellum) of untreated diabetic rats was observed in the study (Table 16, 17 and Figure 22, 23). In our present study, we tried to measure the combined antioxidant effect of the non-enzymatic defences present in the brain tissue homogenates by FRAP method, which measures the ferric reducing ability of brain tissue homogenate. The non-enzymatic
antioxidants such as ascorbic acid, Trolox, \( \alpha \)-tocopherol, in a mixture with olgalbumin, uric acid and bilirubin act as reductants and inactivate the oxidants by redox reactions \((401)\). We found a significant decrease in the FRAP values in the tissue homogenates of hippocampus, frontal cortex and cerebellum of the untreated STZ rats in comparison to the controls. Decrease in the antioxidants in the brain regions of the diabetic rats could be one of the pathophysiology of cognitive decline in the diabetic rats. On treatment with the omega-3 PUFA and vitamin C and B\(_{12}\), a marked increase in the FRAP values from the different brain areas are observed. \((\text{Table 17} & \text{ Figure 23})\) The results confirmed the antioxidant property of the omega-3 PUFA present in the fish oil and the flaxseed oil. High dose of fish oil and flaxseed oil in combination with vitamin C had the best effect in increasing the FRAP values of the different brain areas.

To the best of our knowledge the present study is the only study to have examined the association of oxidative status and cognitive functioning in STZ induced diabetic rats undergoing dietary supplementation with both the sources of omega-3 at different doses. Among the key findings are the positive correlation of protein carbonyl and PT\(_{-1}\) (time taken by the animal to reach the target quadrant), indicating that an increase in oxidative stress is associated with loss of memory and hence the animals take longer time to get into the target quadrant. \((\text{Table 15.2} & \text{ Figure 21.1})\)

There was negative correlation between GSH (Frontal Cortex), FRAP (Cerebellum), FRAP (Frontal Cortex), FRAP (Hippocampus) with PT\(_{-1}\) (time taken by the animal to reach the target quadrant), signifying an improved memory in the study group of animals. Association of antioxidant status with enhanced memory can also be depicted by considering the positive association of GSH (Frontal Cortex), FRAP (Cerebellum), FRAP (Frontal Cortex), FRAP (Hippocampus) with PT\(_{-2}\) (time spent by the animal in the target quadrant) during the probe trial of MWM test. \((\text{Table 17.1} & \text{ Figure 24, 24.2, 25, 25.1, 26, 26.1, 27, 27.1})\)

Considering that cognitive performance could be affected by the oxidative status multiple linear regression analysis was carried out. In the present study protein carbonyl was the only oxidant to be significantly correlated with memory decline. Frontal cortex GSH and hippocampal FRAP levels were found to be the best predictor
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of cognitive performance, demonstrating their involvement in maintenance of spatial memory. (Table 20)

7.4. Effect of omega-3 PUFA rich oils and vitamin C and B12 on Brain derived neurotrophic factor (BDNF) of different brain areas

Hyperglycaemia affects the neuronal cell number and anatomy of the hippocampus and hypoglycaemia leads to neuronal death particularly in the dentate gyrus and CA1 regions of hippocampus. (528) In a recent study it has been reported that T2DM patients have lower serum BDNF levels. (529) They found a positive correlation between serum BDNF level and delayed memory, postulating a lower serum BDNF levels in T2DM patients as a risk factor for cognitive dysfunction. In T2DM BDNF has multiple roles other than neuroprotective function, it has been implicated in the regulation of blood glucose and HbA1c when administered weekly once or twice for 3 weeks. BDNF also increases the pancreatic insulin content and protects β cells, enhances norepinephrine turnover and thermogenesis. All the above-mentioned role of BDNF indicates that it stimulates the sympathetic nervous system and hence regulates the metabolism in obese and diabetic animals. (530,531) In fact, BDNF has been referred as ‘metabokine’ due to its effect on glucose, lipid and energy balance. As BDNF can cross the blood brain barrier, (532) serum BDNF levels have been correlated with that of brain BDNF (533) and measuring the circulatory levels could be taken as a predictor of cognitive dysfunction. Clustering of epidemiological studies have shown an implication of reduced BDNF levels and depression in T2DM patients. (180) Animal studies with STZ rats have proved that T1DM manifests with arrays of neurodegenerative changes for which reduction in BDNF levels could be considered as one of the major reasons. (407) Lack of BDNF in the adult mice hippocampus causes hippocampal dysfunction while in developing brain it causes hyperactivity and defect in hippocampal dependent learning. (534)

In the present study, we observed a decrease in hippocampal BDNF levels in the diabetic control rats as compared to the normal controls; the findings are similar to other studies. (183,407,535) (Table 18 & Figure 28)

A significant negative correlation between hippocampal BDNF and time taken by the animal to reach the target quadrant of MWM indicated a better memory with
increased BDNF levels. (Figure 30) Treatment with omega-3 PUFA rich oils and vitamin C and B12 improved the hippocampal BDNF levels and was accompanied with an improvement in the spatial memory as assessed by MWM and passive avoidance test. Several of the studies have reported enhanced hippocampal neurogenesis in parallel to the increased BDNF following the treatment with omega-3 PUFA. (536–538) Omega-3 PUFA enriched diet also increases the pro BDNF along with mature BDNF, which gets boosted up additionally with the inclusion of exercise. (352) In addition, in DHA enriched diet there is an increment of activated form of CREB and synapsin I, hippocampal Akt and CaMKII, which are critical for learning and memory and synaptic plasticity and these effects are enhanced with exercise. (352) Plasma level of BDNF is increased with oral consumption of α-linolenic acid in healthy adult humans, (539). Considering that the circulatory BDNF reflects the brain BDNF level, we can propose that in the present study, flaxseed oil rich in ALA might have augmented the expression of BDNF in the hippocampus of the flaxseed oil treated group of animals. (Table 18)

Association of low serum BDNF with cognitive impairment in various neurodegenerative diseases are contradictory. While several lines of evidences have proved that serum BDNF are significantly low in patients with cognitive decline associated diseases, such as Huntington’s disease, (540) Schizophrenia, (541) Alzheimer’s disease, (542) and Mild Cognitive Impairment. (543) There are few other lines of evidences which contradict the above findings and have shown that there is compensatory increase in serum BDNF in AD and MCI (Mild cognitive Impairments), (544,545).

Thus, citing all these results it can be proposed that omega-3 PUFA enhances synaptic plasticity, neural growth and spatial learning and memory by enhancing the BDNF expression in different brain regions.
7.5. Effect of omega-3 PUFA rich oils and vitamin C and B\textsubscript{12} on Tumor necrosis factor-alpha (TNF-alpha) of different brain areas

In CNS, TNF-\(\alpha\) is the principal proinflammatory cytokine mediating the neuroinflammatory changes leading to cascade of events which ultimately leads to the neuronal death. The other factors that mediate neuroinflammation include cytokines, chemokines, free radicals, prostaglandins, anaphylotoxins, proteases, pentraxins, complement components, and adhesion molecules. (546) Individuals with diabetes and insulin resistance are reported with an elevated proinflammatory cytokine levels such as TNF-\(\alpha\), IL-6, and C-reactive protein. (547) Studies on animal and humans have reported the interrelationship between insulin resistance, inflammation and diabetes. Inflammatory changes in the adipocytes contribute to the systemic low grade inflammation leading to insulin resistance and eventually lead to diabetes. (548,549) A study with diabetic and obese mouse (db/db) showed impaired spatial memory with elevated inflammatory cytokines (IL-1\(\beta\), IL-6 and TNF-\(\alpha\)) and reduced BDNF in the hippocampus. (124) In the present study, we had seen an increase in the TNF-\(\alpha\) level in the hippocampus of the STZ induced diabetic rats as compared to the control rats. Treatment with omega-3 PUFA and vitamin C and B\textsubscript{12} showed anti-inflammatory property and reduced the TNF-\(\alpha\) level in the diabetic rats. High dose of flaxseed oil given along with vitamin C and B\textsubscript{12} showed the lowest levels of hippocampal TNF-\(\alpha\) in comparison to all the other sets of treatment. (Table 18, Figure 29) There was negative correlation between hippocampal TNF-\(\alpha\) and cognitive parameter of Morris water maze. Animals with higher values of TNF-\(\alpha\) had lesser memory retention and spent lesser time in the target quadrant during probe trial of MWM test. (Figure 31) TNF-\(\alpha\) level in the brain is increased following any brain injury, infection, or neurodegenerative and neurotoxic conditions. AD patients have high levels of TNF-\(\alpha\) in the amyloid plaques while in PD patients there is increased expression of TNF-\(\alpha\) in the caudate and putamen. (176) TNF-\(\alpha\) mediates its neurotoxic effects by the following mechanisms:- a) by promoting infiltration through the BBB and adhesion of immune cells on the injured cells; b) by stimulating apoptosis of the brain microvascular endothelial cells; c) initiating a vicious cycle of oxidative outburst and
release of inflammatory cytokines; d) preventing glutamate intake and potentiating glutamate mediated toxicity; e) increasing the vasogenic brain edema; f) modulating calcium homeostasis and ion currents; g. and lastly regulating the LTP and membrane potential. (550)

Thus, TNF-\(\alpha\) plays the role of central facilitator of neuroinflammation and brain injury. It also has neuroprotective effect where it causes the release of neurotrophic factors, activates astroglia, promotes the survival of oligodendria, stimulates synaptic currents and mediates neuronal plasticity, activates NF-\(\kappa\)B pathway, induces anti-apoptotic factor and antioxidant defence pathways. (550)

Findings from many of the studies have focussed that probably inflammation contributes towards the development and progression of depression by affecting the neuroplasticity via the reduction of BDNF. (551)

We observed a correlation between TNF-\(\alpha\) and BDNF, rats with higher proinflammatory marker in the hippocampus had lesser BDNF in the hippocampus. Our results are in accordance with other studies. (124,551) (Figure 31.1) The interplay between inflammation, neurogenesis and neurodegeneration is more complex as expected. In a recent report, it has been shown that lipopolysaccharide (LPS) induced anxious and depressive behaviour in the rats are associated with decreased hippocampal BDNF expression. (552) Even in stress conditions there is decrease in BDNF level in CA1 and CA3 areas of hippocampus (553) and the possible mechanism proposed for this decrease in BDNF is stimulation of HPA axis by the proinflammatory cytokines. (554) Glucocorticoids are thought to reduce the levels of BDNF. (184)

In the current study, we observed a decrease in hippocampal TNF-\(\alpha\) level with supplementation of omega-3 PUFA and vitamins. Evidences from other studies have also indicated that omega-3 PUFA reduced the proinflammatory cytokines, adhesion molecules and COX-2 by inactivating the NF\(\kappa\)B system. Anti-inflammatory action of omega-3 PUFA can be attributed to many ways such as decreased formation of omega-6 derived inflammatory eicosanoids, enhanced production of anti-inflammatory lipid mediators from EPA and DHA. (556–558) These together suppress the activity of NF\(\kappa\)B (via AMPK/SIRT1 pathway), an important transcription factor involved in the upregulation of the genes encoding the inflammatory cytokines
Discussion

(including COX-2, TNF-α and IL-1B) and adhesion molecules. Hence, this could be the suggested mechanisms for the anti-inflammatory action of omega-3 PUFA supplementation in our study.

In our study, we did not observe any anti-inflammatory action of vitamin C and vitamin B₁₂ independently in our rat models rather we observed a synergistic effect of their combination with either fish oil or flaxseed oil in different doses. A recent study has reported the potential of vitamin C (500 mg twice daily) as an anti-inflammatory agent in hypertensive and obese diabetic patients (559) which could not be replicated in our experimental study. A recent study on AD has shown that low level of epidermal growth factor and high level of TNF-α with vitamin B₁₂ deficiency has the pathophysiological role in the development of dementia. (560) Supplementation of omega-3 along with vitamin B₁₂ and folic acid has a beneficial effect on the oxidative stress and inflammation in preeclampsia, (561) but studies on the combined effect of omega-3 PUFA and vitamin B₁₂ on the cognition of STZ diabetic rats are scarce. Studies have also reported that for the optimal neuroprotective function of omega-3 PUFA, a sufficient vitamin B status with low homocysteine is the prerequisite. (562)

In the present study, we used groups with multiple combination of supplements of fish oil and flaxseed oil in low and high dose along with the vitamin C and vitamin B₁₂ combination to evaluate an efficient combination of dietary supplement that would help in prevention and treatment of cognitive dysfunctions in diabetic patients. There was an overall significant beneficial effect on lipid profile, spatial memory, exploratory behavior, anxiety and fear behavior, oxidative status, hippocampal inflammation and neuroprotection. These findings indicate that omega-3 PUFA from fish oil and flaxseed oil have significant neuroprotective effect in the brain tissues, making it an effective nutrient for reducing impairments to learning and memory abilities caused by diabetes.