

Fish Oil Supplementation Ameliorated Brain Lesions Induced by Diabetes and Hypercholesterolemia in Male Wistar Albino Rats

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Abstract

Diabetes and hypercholesterolemia are dyslipidemic diseases and have certain role in brain dysfunction, but little of works are concerning with it. In the present study we used eighty male Wistar rats weighing approximately 100 ±15 gram. The animals were arranged into 8 groups; Control (C), fish oil-treatment, hypercholesterolemic group (H), hypercholesterolemic & fish oil-treatment (HF), diabetic- group (D), diabetic and fish oil-treatment (DF), combined hypercholesterolemic and diabetic group (HD) and combined hypercholesterolemic and diabetic group and fish-oil-treatment (HDF). Diabetes was induced by streptozotocin (40mg/kg single dose in citrate buffer pH4.6). Hypercholesterolemia was carried out by feeding rats on diet containin3% cholesterol. Fish oil (Menhaden, Sigma-aldrich, highest purity) was supplemented orally every other day at 100mg/kg body weight. Treatment was carried out for 16 weeks. At the end of treatment, brain tissues were subjected for histological investigation and biochemical assessments of dopamine, serotonin, vascular endothelial growth factor, 8-deoxyhydroxy-guanosine, adhesion molecules and phospholipids beside histological investigation of cerebral hemisphere and cerebellum. The present finding revealed marked depletion of the assayed neurotransmitters and phospholipids and increased of vascular endothelial growth factor, adhesion molecules and 8-deoxy hydroxy-guanosine. Histological observations of cerebral hemisphere revealed widespread of hemorrhagic spots in hypercholesterolemia, neovascularization in combined diabetes and hypercholesterolemia and dense lymphocytic infiltration in diabetic group. All the experimental groups possessed edematous lesions in the inner plexiform layer. Cerebellar cortex exhibited massive degeneration of Purkinje cells and granular cell layer in diabetic and or hypercholesterolemia. Fish oil supplementation improved the brain function and histological picture. The authors concluded that fish oil contain short and long chain omega-3 fatty acids fatty acid which support the brain function and scavenge the free radicals damaging brain cells.

Keywords: Cerebral Hemisphere; Cerebellum; Brain Function; Diabetes; Hypercholesterolemia; Fish Oil

Introduction

The prevalence of diabetes was markedly increased throughout the world, reached approximately to 285 million, 90% of them possessing type2 cases. In 2013, 381 million people had diabetes according to International Diabetes Federation. The prevalence of the disease is markedly progressed and the number being almost double by 2030 [1,2]. On the other hand, hypercholesterolemia and obesity were associated with 50% of the mortality caused by ischemic heart disease and stroke, and reached almost from 3% to 53% in men, and from 4% to 40% in women as well as over 50% of men and women taking lipid-lowering drugs had abnormal cholesterol level [3-5]. Both diseases are associated with the development of neurotoxicity and Alzheimer's disease (AD) [6].

Neurons required high glucose level for their high metabolic rate and this depend on the extracellular level of glucose. Consequently, hyperglycaemia was interfered with the brain function [7]. Type 2 diabetes model ob/ob mice was found to exhibit impaired insulin expression in pancreas, liver and midbrain as well as over-expressed α -synuclein and endoplasmic reticulum stress markers (CHOP and GRP78) in pancreas and midbrain, followed by increased production of inflammatory mediators such as interleukins IL-1 β leading to the development of the etiology of Parkinson's disease (neurodegenerative disease) [8].

In vitro studies of incubated murine Schwann cells (IMS32) with 30 and 56 mM glucose for 48 h and 7 and 14 days led to increase of lipid peroxidation, aldose reductase level and caspase 3, the markers of neuronal damage [9]. Diabetic rats was found to possess decreased glucose levels associated with increased mitochondrial damage via

altering both cytochrome c and phosphatidylserine leading to DNA damage [10]. It is known that the mitochondrial system is a main source of cellular reactive oxygen species. The production of H₂O₂ is affected by the mitochondrial membrane potential and oxygen consumption. Altered insulin secretion led to oxidative neuronal damage and neurodegeneration [11]. Hyperglycemia was found to cause mitochondrial fragmentation and production of reactive oxygen species, the major complications associated with diabetes and obesity [12,13]. It is well known that astrocyte is the main neurovascular unit involved in brain stroke. Oxygen-glucose deprivation was found to alter the expression/distribution and activity of glial glutamate transporters, impaired and affect intracellular levels of glutathione in differentiated astrocytes [14]. Astrocytes are the main source of apolipoprotein E (apoE) comparing with oligodendrocytes, microglia, and ependymal cells which protect it against glutamate induce cell damage and mediate cholesterol homeostasis in the brain, however disrupted cholesterol metabolism led to overexpression ApoE mRNA levels mediating the development of Alzheimer's disease (AD) [15,16]. There are several factors involving in the development of AD include

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hypercholesterolemia, hypertension, obesity, and diabetes [17]. Hypercholesterolemia was found to be associated with altered brain cholesterol metabolism and formation of oxysterols, which cross the blood brain barrier and increased neuroinflammation and subsequently increase extracellular deposits of amyloid β in the cerebral blood vessels, and intracellular inclusions of hyperphosphorylated tau, the markers of AD [6,18,19]. Alzheimer's disease (AD) is characterized by the progressive loss of neurons and synapses, and extracellular deposits of amyloid β (Ab) in the cerebral blood vessels, and intracellular inclusions of senile plaques of neurofibrillary tangle [19]. Hypercholesterolemia is closely related to cardiovascular disease and consequently interfered with hippocampus, whole brain, ventricle, middle temporal lobe, fusiform, and entorhinal volume loss in patients with AD and cognitive function [20-22]. Diabetes mellitus, atherosclerosis and hyperlipidemia represent the main risk factors for cerebrovascular accidents [23].

Fish oil contains the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), precursors of certain eicosanoids [24].

Dietary supplementation of eicosapentaenoic acid (EPA) appeared to resolute the risk of recurrent stroke in a Japanese population of hypercholesterolemic patients [25].

There is a dearth of information regarding the contribution of diabetes and hypercholesterolemia in brain disease. The present study aimed to illustrate the cerebral and cerebellar disruption and markers involved in neuronal cell damage and explained the role of fish oil in amelioration.

Materials & Methods

Induction of diabetes

Experimental diabetes mellitus was carried out by a single intraperitoneal injection of streptozotocin (40 mg/kg) in citrate buffer (0.05 M) (pH 4.6) [26]. Treatment was carried out for 16 weeks. Control animals were treated with physiological saline as vehicle. Hyperglycemia was verified by measuring the blood glucose within the range of 180-220 mg/dL.

Induction of hypercholesterolemia

The experimental group was fed a hypercholesterolemic diet composed of 3% cholesterol and 15% cocoa butter and 0.2% cholic acid and 0.2% thiouracil for 16 wks [27]. The control group was fed on a standard diet free from hypercholesterolemic components.

Fish oil-supplementation

Fish oil (Menhaden, Sigma-aldrich, highest purity) was used in the experimental work. Each rats received oral doses of 100mg/kg body weight (b.wt.) every other day during the period of treatment.

Experimental work

Fifty male albino rats weighing approximately 100 \pm 15g body weight, obtained from Breeding Farm, Ministry of Health, Giza, Egypt. They were fed on standard diet and water was allowed ad libitum throughout the experimental period. The animals were housed in good ventilation with 12 hour light and dark cycle. Male rats were fed on hypercholesterolemic diet for 16 weeks. Diabetes was carried out and animals allowed to feed on standard diet for 16 weeks. Rats were arranged into four groups (n=10) such as Control (C), Fish oil-treatment, hypercholesterolemic-group (H), hypercholesterolemic & fish oil-treatment (HF), diabetic- group (D), diabetic and fish oil-

treatment (DF), combined hypercholesterolemic and diabetic group (HD) and combined hypercholesterolemic and diabetic group and fish-oil-treatment (HDF). At the end of treatment, male rats of both control and experimental groups were anaesthetized by ether and sacrificed. Head regions were dissected and brains were removed and subjected for the following investigations:

Histological investigation

Brain of both control and experimental groups and fixed in 10% phosphate buffered formalin (pH 7.4). They were dehydrated in ascending grades of ethyl alcohol, cleared in xylene and mounted in molten paraplast at 58-62°C. Five micron sections were carried out, stained with hematoxylin & eosin and investigated for histopathological changes under a bright field light microscope.

Biochemical Assessments

Determination of neurotransmitters

Whole brain tissue of both the control and experimental groups were homogenized in Tris buffer at pH 7.5 and separated their supernatant and stored in refrigerator. 5-hydroxytryptamine and dopamine (DA) were determined fluorometrically, according to Schlumpf et al. (1973) [28].

Vascular endothelial growth factor (VEGF) and adhesion molecules (ICAM-1 and VCAM-1)

Vascular endothelial growth factor and adhesion molecules were determined by Quantikine ELISA kit of R&D System, Minneapolis, MN, USA . according to the manufacturer's protocol. Absorbance was measured at 450 nm by an ELISA plate reader UV-Spectrophotometer (Perkin Elmer Victor 3™) with Software Wallac 1420 version 3.0.

8-hydroxy-2-deoxy guanosine (8-OHDG)

The amount of 8-OHDG in brain tissue of both control & experimental groups was determined using the Bioxytech ELISA Kit (OXIS Health Products, Portland, OR, USA) according to the manufacturer's instructions. The reaction was terminated and the absorbance was measured using a FLUO star OMEGA microplate reader (BMG LABTECH Ltd., Germany) at a wavelength of 450 nm [29]. Values of 8-OHDG are expressed as mg/ml.

Phospholipids

Brain phospholipid contents (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin) were determined according to Bligh and Dyer (1959) [30]. A known weight of brain was thoroughly mixed with a known volume of methanol and chloroform at a ratio of 2:1 and a known volume of deionized water. The mixture was homogenised and centrifuged for 5 min at 4000 rpm. The supernatant was collected and remixed with chloroform and methanol at a ratio of 2:1. The methanol: water phase was sucked up with a water stream pump. Under a stream of nitrogen, the CHCl₃ - phase was evaporated to dryness. The crude lipids were redissolved in 0.4 ml CHCl₃: MeOH (2:1 v/v) and transferred into a capped test tube and filtered. Fifty μ L of the filtrate were injected into the chromatographic system.

Statistical analysis

Data were presented as mean \pm standard error. The statistical analysis was performed with multi-variant analysis of variance (MANOVA) using SPSS (version 13) software package for windows

	DA. (ng/mg)	5-HT (ng/mg)	VEGF (ng/100mg)	8-HDG (ng/100mg)	ICAM-1 (ng/100mg)	VCAM-1 (ng/100mg)	PTC (nmol/mg)	PHE (nmol/mg)	PS (nmol/mg)	SM (nmol/mg)
C	90.6±1.1	497.1±5.1	137.1±6.3	1.4±0.1	2.5±0.2	2.6±0.2	0.4±0.1	1.4±0.1	0.4±0.03	0.8±0.1
F	91.6±1.4	506.4±5.0	140.2±5.82	1.5±0.1	2.4±0.2	2.88±0.26	0.44±0.04	1.33±0.1	0.4±0.1	0.8±0.1
D	67.1±2.9	410.3±4.8	146.9±5.4	3.0±0.2	3.45±0.2	4.3±0.2	0.4±0.02	0.8±0.1	0.3±0.02	0.4±0.03
DF	74.1±2.1	451.4±2.1	160.1±2.3	1.7±0.2	4.1±0.3	4.9±0.4	0.4±0.05	1.0±0.1	0.3±0.02	0.5±0.03
H	61.1±1.8	417.0±6.4	140.8±1.9	2.6±0.1	3.3±0.2	4.1±0.27	0.4±0.02	0.9±0.1	0.22±0.02	0.42±0.02
HF	71.1±2.1	462.5±4.7	164.7±3.2	1.8±0.2	4.0±0.1	4.9±0.2	0.4±0.04	1.1±0.1	0.3±0.03	0.63±0.01
HD	55.2±2.3	385.4±5.1	176.5±3.2	3.6±0.2	3.4±0.3	4.0±0.3	0.4±0.03	0.7±0.1	0.2±0.04	0.4±0.02
HDF	66.1±2.7	435.3±3.2	141.2±3.4	2.4±0.4	3.5±0.2	4.4±0.4	0.47±0.03	1.1±0.3	0.3±0.06	0.6±0.01
<i>F-test</i>	38.42	31.85	24.78	6.01	5.52	4.25	1.98	3.57	3.97	10.48
<i>P<0.05</i>	S	S	S	S	S	S	IS	S	S	S
	DA. (ng/mg)	5-HT (ng/mg)	VEGF (ng/100mg)	8-HDG (ng/100mg)	ICAM-1 (ng/100mg)	VCAM-1 (ng/100mg)	PTC (nmol/mg)	PHE (nmol/mg)	PS (nmol/mg)	SM (nmol/mg)

Table 1: Role of fish oil in improved biochemical parameters of brain tissues affected by diabetic and or hypercholesterolemic rats. Each result represent M±SE (n=5); C, control; F, fish oil; D, diabetes; DF, diabetes & fish oil; H, hypercholesterolemia; HF, hypercholesterolemia and fish oil; HD, hypercholesterolemia and diabetes; HDF, hypercholesterolemia and diabetes and fish oil; 5-HT, serotonin; VEGF, vascular endothelial growth Factor; 8-HDG, 8-hydroxyguanosine; ICAM-1, intracellular adhesion molecule; PHE, phosphatidylethanolamine; PS, phosphatidylserine; PTC, phosphatidylcholine; SM, sphingomyelin; VCAM-1, vascular cell adhesion molecule, IS, Non-significant at P < 0.05, S, Significant at P < 0.05.

for comparing the multivariations between control and experimental group. P<0.05 was considered statistically significant.

Results

Biochemical observations

From table (1), treatment with diabetes and or hypercholesterolemia revealed marked reduction of the neurotransmitters dopamine and serotonin. However, fish oil supplementation restored the level of both assayed neurotransmitters and adhesion molecules but attain the normal levels. Concerning intracellular and vascular adhesion molecules (ICAM-1& VCAM-1), VEGF and 8-HDG, their levels were markedly increased in brain tissues of diabetes and or hypercholesterolemia and ameliorated after fish oil supplementation but still not matched with the control values. The assayed phospholipids (phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin) were markedly depleted in brain of diabetes and or hypercholesterolemia and retained nearly normal level after fish oil supplementation.

Histopathological Observations

Cerebral hemisphere

In control, the cerebral hemisphere possessed normal arrangement of molecular, outer granular, outer pyramidal, inner granular, inner pyramidal and multiform cellular layer. Fish-oil supplementation exhibited similar histological picture (Figure 1: A-C).

In hypercholesterolemic group, there was a detected hemorrhage area enclosed the peripheral sheath of cerebrum. There was a marked reduction of the outer granular cells associated with increased average of cells with pyknotic nuclei. Edematous lesions were distinguished in the inner granular and inner plexiform layer. Most of their cells showed vacuolar degeneration (Figure 1: 1B&B1).

Diabetic group exhibited massive fragility and loss neural fibers on the molecular layer with subsequently accumulation of inflammatory cells. Gliosis necrotic lesions were detected in inner-plexiform layer (Figure 1: C&C1). Diabetes and hypercholesterolemic group exhibited dense neovascularization around the cerebral capsule. Molecular layer possessed reticular structural- pattern and outer granular layer possessed increased average of neuronal cells with pyknotic nuclei. Edematous lesions were detected in both the inner granular and inner

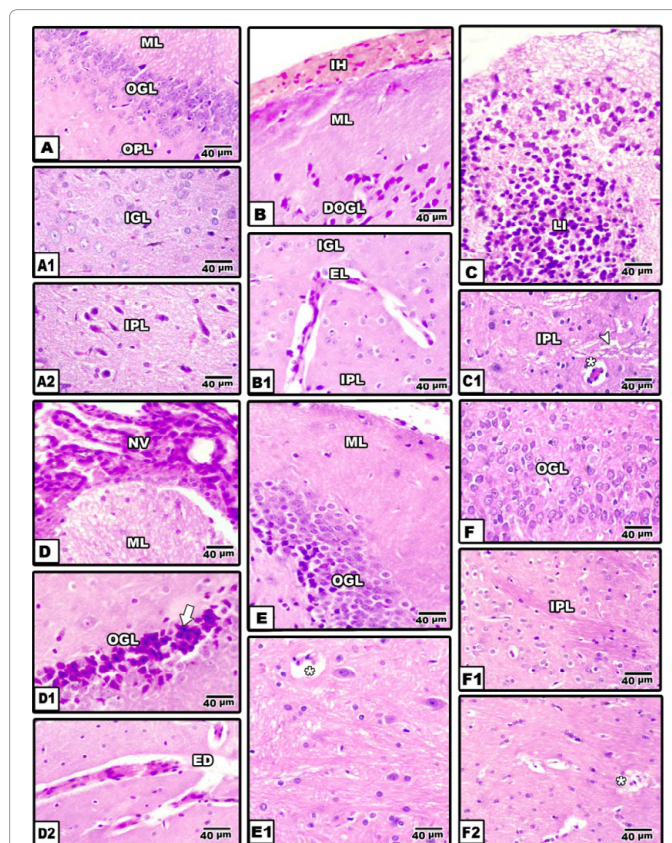


Figure 1: Photomicrographs of sagittal sections of cerebral hemisphere of male rats. A-A2: Control showing normal pattern of cerebral cell layers. B-B1: Hypercholesterolemia showing cerebral hemorrhage with damaged outer granular cells and edematous inner plexiform and inner granular layer. C-C2: Diabetic showing dense collection of inflammatory cells in the molecular and outer granular layer and necrotic foc in the inner plexiform one. D-D2: Diabetic and hypercholesterolemia showing dense neovascularization peripherally coating the outer periphery. Note increased average of damaged outer granular cells and edematous lesions in inner plexiform. E-E1: Diabetic. F-F2: Diabetic and Hypercholesterolemia. Note improvement of histologic picture. DOGL, degenerated outer granular; ED, edematous lesions; IGL, Inner granular layer; IH, internal hemorrhage; IPL, inner plexiform layer; LI, lymphocytic infiltration; ML, molecular layer; NV, neovascularization; OGL, outer granular layer; OPL, outer plexiform layer. H&E.

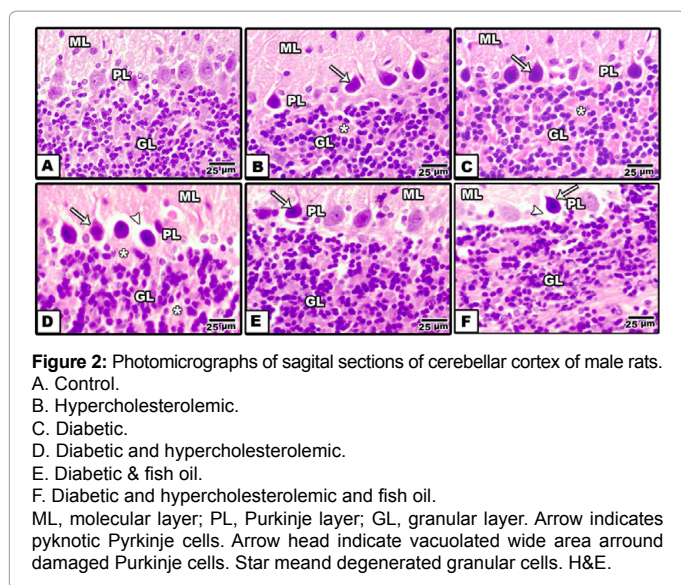


Figure 2: Photomicrographs of sagittal sections of cerebellar cortex of male rats. A. Control. B. Hypercholesterolemic. C. Diabetic. D. Diabetic and hypercholesterolemic. E. Diabetic & fish oil. F. Diabetic and hypercholesterolemic and fish oil. ML, molecular layer; PL, Purkinje layer; GL, granular layer. Arrow indicates pyknotic Purkinje cells. Arrow head indicate vacuolated wide area around damaged Purkinje cells. Star meand degenerated granular cells. H&E.

plexiform layer (Figure 1: D-D2). Diabetes and or hypercholesterolemia which received fish oil-supplementation revealed marked improvement but was still possessed slight pathological changes on outer granular cells which showed pyknotic nuclei as well as vacuolar degenerated neurons and mild necrotic foci in both the inner granular and inner plexiform layer (Figure 1: E-E1 and F-F2).

Cerebellum

In normal cerebellum, three different cell layers were distinguished including molecular, Purkinje and granular cell layer. The cells exhibited peculiar normal pattern structure and arrangement (Figure 2: A). In experimental diabetic and or hypercholesterolemia, there was a marked increase of Purkinje cell loss. Most of them appeared darkened with pyknotic nuclei. Widened spaces aroused around Purkinje cells in experimental diabetic and hypercholesterolemic group. The cell densities of the granular cells were markedly reduced and some of them become eosinophilic (Figure 2: B-D). Fish oil supplementation to diabetes and or hypercholesterolemic group improved the histopathological pictures but a least average Purkinje cells were still with pyknotic nuclei (Figure 2: E & F).

Discussion

Neuron cell function and viability is mediated by a constant oxygen and glucose supply [7,31]. The brain tissues needed continuous supply of glucose and oxygen. These requirements in parallel with regular cerebral blood flow, cerebral oxygen delivery, and normal mitochondrial function are important for the maintenance of brain function and tissue viability [32]. Reduced supply of glucose led to many brain diseases such as stroke and ischemic injuries [33].

From the present findings diabetes and or hypercholesterolemia led to marked impairment of brain function assessed by depletion of both the neurotransmitters dopamine and serotonin.

Similar findings of decreased DA transporter expression in the nucleus accumbens in rat fed on high fat diet [34]. Serotonin and dopamine (DA) systems are closely related with each other at the neurophysiological function and that impairment of the serotonin system function can altered expression of the dopamine system [35,36].

Altered striatal dopamine functions were detected in obese OLETF

rat [37]. Also, 5-HT overexpressed in normal pancreas insulin secretion and apparently inhibited on diabetic pancreatic tissues [38].

The apparent depletion of serotonin and dopamines appeared to be responsible for impairing brain function. Seo et al. (2008) mentioned that serotonin hypofunction may predispose individuals to impulsive aggression [39].

Also, the present findings revealed apparent cerebral hemorrhage in hypercholesterolemic group and dense cerebral neovascularization in diabetes and or hypercholesterolemia coincides with increased intracellular and vascular adhesion molecules (ICAM-1& VCAM-1) and vascular endothelial growth factor (VEGF). It is known that VEGF is an endothelial cell-specific angiogenic.

Similar findings of diabetes associated cerebral neovascularization and overexpression of VEGF were reported by Prakash et al. (2012, 2013) [40,41]. The author detected increased level of VEGF in cerebral microvessels of diabetic Goto-Kakizaki model of type 2 diabetes parallel with increased cerebral neovascularization.

Sasani, et al. (2011) reported endothelial cell desquamation, and a reduction of waves in the endothelial layer in rabbits fed on hypercholesterolemic diet [42].

The diabetic and hypercholesterolemia stress were associated with intracranial atherosclerotic disease and a decrease of cognitive function [16,43]. Diabetic rats were found to increase ICAM-1 mRNA and protein levels in the frontal cortex [8]. Similar to VEGF, VCAM-1 is an endothelial cell membrane glycoprotein overexpressed in brain during inflammation. Both adhesion molecules were found to be increased in brain metastasis [44]. The observed increase of adhesion molecules and VEGF seemed to be parallel with the detected cerebral hemorrhage and neovascularization in diabetic and or hypercholesterolemia.

Also, there was a marked increase of cerebral cell death especially in the outer granular layer associated with widespread of edematous lesions in inner plexiform and both granular cell layers of diabetic and or hypercholesterolemia. These was confirmed by increased level of 8-hydroxyguanosine in brain. Similar findings of increased 8-hydroxyguanosine in fatal brain edema resulted from type 1 diabetes mellitus [45]. Ding et al. (2015) observed severe ischemic vascular damage in the T2DM rats after stroke [46].

According to Kwee and Nakada (1988), phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin represent the most abundant phospholipids of brain cell membrane [47]. Phospholipids may also involve in transmission and relay signals from the membrane to intracellular compartments or to other cells. Depletion of membrane lipids may contribute to the pathogenesis of depression and anxiety disorders [48]. The assayed phospholipids (phosphatidylethanolamine, phosphatidylserine and sphingomyelin) were markedly depleted in brain of diabetes and or hypercholesterolemia and retained nearly normal level after fish oil supplementation. Brain phospholipid fatty acid 22:5n-6 acid was found to be increased by the high-fat diet in both non-diabetic and diabetic mice [49]. Diabetic rats were found to exhibit depletion of microsomes phospholipid contents of brain [50].

On the other hand, fish oil supplementation to diabetic and or hypercholesterolemic rats led to improvements of the brain neurotransmitters (DA & 5-HT), VEGF, adhesion molecules (ICAM-1, VCAM-1), 8-HDG and phospholipid contents. This t was parallel with apparent amelioration of the histological pictures of the cerebral hemisphere and cerebellar cortex.

Women and experimental mice received dietary supplementation with n-3 PUFA-enriched fish oil revealed significant increase levels of n-3 PUFAs in the brain and improved long-term behavioral outcomes after cerebral ischemia [12,51].

Young adult male rats received transient middle cerebral artery occlusion and received intravenous administration of PUFA n3 revealed inhibition of ischemia-induced increase of cyclooxygenase 2, hypoxia-inducible factor 1alpha, inducible nitric oxide synthase, and interleukin 1beta [52].

Witte et al. (2014) reported that fish oil improved white matter microstructural integrity and gray matter volume in frontal, temporal, parietal, and limbic areas primarily of the left hemisphere, and on carotid intima media thickness and diastolic blood pressure [53].

Fish oil contains long-chain fatty acids: docosahexaenoic acid and eicosapentaenoic acid which have direct effects on reducing the inflammatory state by reducing IL-6, TNF- α , CRP and many other factors [54].

Finally the author concluded that fish oil supplementation improve the histopathological picture and brain function of rats subjected to diabetes and or hypercholesterolemia.

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