

Original article

Docosahexaenoic Acid Modulates Oxidative Stress and Monoamines Levels in Brain of Streptozotocin-Induced Diabetic Rats

Sahar Mohamed Mahmoud¹, Yasmin Abdel Latif², Hisham Orban²,
Amr Mahmoud Ibrahim², Jihan Hussein²

¹Zoology Department, Faculty of Science, Cairo University, Cairo, Egypt

²Medical Biochemistry Department, National Research Centre, Cairo, Egypt

SUMMARY

The prevalence of diabetes mellitus (DM) is increasing in many countries. A lower prevalence of DM type 2 and other glucose metabolism disorders was observed in populations consuming larger amounts of n-3 polyunsaturated fatty acids, existing mainly in fish. Docosahexaenoic acid (DHA) is an important signaling molecule required for the central nervous system continuous maintenance of brain functioning. The aim of this research is to highlight the role of DHA in controlling glycemic measures and modulating the oxidant/antioxidant status and levels of neurotransmitters in brains of diabetic rats. Diabetes was induced with a single s.c. injection of streptozotocin (STZ) (6.0 mg / 0.5 ml /100 g body weight). Experimental male Wister rats (n = 40) were randomly divided into four groups: control group, DHA, STZ-diabetic, and STZ + DHA. All rats were decapitated after 30 days to evaluate glucose and insulin levels, brain oxidative stress and also to estimate monoamines levels. DHA administration significantly improved fasting blood glucose and insulin levels compared to the DHA+STZ group and decreased 8-hydroxy-2'-deoxyguanosine level in their urine. In addition, DHA treatment to STZ-treated rats showed a decrease in malondialdehyde content and advanced oxidation protein product and significantly increased glutathione content in brains of DHA + STZ-treated rats, and decreased the level of monoamines in rat's brain. To conclude: DHA modulated the elevated oxidative stress and neurotransmitters levels, and also acetylcholinesterase activity in diabetic rat brain via enhancing insulin level in serum.

Key words: diabetes, docosahexaenoic acid, oxidative stress, neurotransmitters, insulin

Corresponding author:
Sahar Mohamed Mahmoud
e-mail:Sahar@sci.cu.edu.eg

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder which affects multiple physiological systems including the central nervous system (1). According to WHO, more than 180 million people worldwide have DM and this may double by 2030. DM Type 2 is the most frequent form, constituting 85% of all DM cases, while type 1 DM and specific/gestational DM constitute 10% and 5%, respectively. Studies indicated that clinical complications and morbidity due to DM could be decreased by adjusting glucose level tightly. Both types of diabetes were reported to induce neuronal and brain changes, as those in Alzheimer's disease (2).

The incorporation of sea foods in the human nutrition, especially the polyunsaturated fatty acids (PUFAs) of 20 or more carbon atoms was a significant turning point in human evolution (3). Both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are n-3 common long chain-PUFAs (found in fish, shellfish, micro- and macro-algae and some mammals). DHA deficiency is correlated with several neurological disorders, including Alzheimer's and Parkinson's disease, schizophrenia, and depression (4). Unlike liver, the brain cannot efficiently convert dietary alpha linolenic acid to DHA (5), and is completely dependent on the uptake of DHA from the plasma. DHA was identified as the fundamental neuroprotective fatty acid used against cerebral aging, and neurodegenerative diseases, especially in ischemia (6).

The present study aimed to shed light on the beneficial effect of DHA intake on blood sugar and insulin levels in blood sera as well as 8-hydroxy-2'-deoxyguanosine (8- OHdG) level (as a product of oxidative DNA damage) in urine of STZ-induced diabetic rats, and whether DHA treatment could improve insulin and blood sugar levels in rats' sera together with ameliorating oxidative stress and neurotransmission in the adult rat brain during experimental induction of diabetes.

MATERIALS AND METHODS

Chemicals

Docosahexaenoic acid was brought from Fischer Scientific Company (USA), while streptozotocin was purchased from Sigma (Germany). All lab-

oratory chemicals used for HPLC were of analytical grade, and water provided for all experiments was also of ultrapure quality.

Experimental animals and ethical approval

The animal house of National Research Center (NRC) supplied the male albino rats (180 ± 10 g) used in the study. Animals were acclimatized for 10 days to the experiment as they were kept in stainless steel cages at $22 \pm 2^\circ\text{C}$ and light/dark cycle (12 h/12 h). The guidelines and regulations of the ethical committee of NRC (Ethical No. 16303) were followed in this study.

Experimental design and induction of diabetes

Forty rats were divided into four groups (10 rats in each group): group I, control rats; group II (DHA) in which rats received 10 mg DHA/kg bw/day; group III (STZ), streptozotocin (STZ) was dissolved in 50 mM sodium citrate (pH 4.5) solution containing 150 mM NaCl, and rats had a single s.c. injection of STZ (6.0 mg/0.5 ml/100 g body weight). Fasting blood sugar was checked after 3 days to confirm DM induction in rats (7). Group IV (STZ + DHA) included STZ- injected rats who were orally administered DHA. After 30 days of treatment, urine samples were collected from all rats for 24 h, for 8-OHdG estimation. Rats were overnight fasted, and blood was collected from the orbital vein. All animals were decapitated and the brain was excised immediately and rinsed with ice-cold saline. For determining fasting blood glucose, plasma was separated using cooling centrifuge (Laborzentrifugen, 2K15, Germany).

Tissue Homogenate

Brain was quickly removed, perfused with iced saline to remove blood cells, blotted on filter paper, and finally frozen at -80°C . A 5 ml of cold buffer (0.5 g of Na_2HPO_4 and 0.7 g of NaH_2PO_4 per 500 ml of deionized water) (pH 7.4) were used to homogenize the brain frozen tissue. After homogenization, all brain samples were centrifuged at 4000 rpm for 15 min at 4°C . The supernatant was kept for different biochemical parameters estimation (8, 9).

Biochemical estimations

Fasting blood glucose level and insulin level as well were determined according to the enzymatic colorimetric method of Trinder (10) and the ELIZA technique, respectively.

Determination of brain oxidant/antioxidant stress parameters

Brain malodialdehyde (MDA) was determined according to Ohkawa et al. (11), while reduced glutathione (GSH) was carried out according to Beutler (12) using commercial kits (colorimetric) from Biodiagnostics, Egypt. Using an ELISA kit from NOVA bioneovan Co. Ltd, China, the advanced oxidation protein product level in the brain (AOPP; estimating the degree of oxidant-mediated protein damage) was determined, according to the manufacturer procedure.

Determination of 8-hydroxy-2'-Deoxyguanosine level in urine

By a modified method described previously and according to Hussein et al. (13), an HPLC analysis was carried out to evaluate 8-hydroxy-2'-deoxyguanosine (8-OHdG) content. In brief, ultrapure water was used to dissolve the 8-OHdG standard. Serial dilutions with different concentrations were prepared and injected onto HPLC for drawing a standard curve. 8-OHdG in urine samples were extracted from 1 ml urine using Strata C18-E (55 μ m, 70 Å) column. The eluents were dried under nitrogen gas and then reconstituted in 5 ml ultrapure water. 20 μ l were injected onto HPLC from each sample.

HPLC condition

An acetonitrile/methanol/phosphate (8.8 g of potassium di-hydrogen phosphate (KH_2PO_4) in 1000 pH 3.5) buffer (25/10/965) v/v was used as the mobile phase. The buffer was filtered two times using 0.45 μ m pore size sterile membrane filter at a flow rate of 1 ml/min through HPLC reverse phase column (250 \times 4.6, particle size 5 μ l) with an electrochemical detector of cell potential of 600 mV. The 8-OHdG concentration was calculated from the standard curve and then divided by the urinary creatinine content estimated by a kinetic method (14).

Determination of brain monoamines

Brain serotonin, noradrenaline, and dopamine were determined according to Hussein et al. (15). Analysis was undertaken with Agilent technologies 1100 series HPLC system, with a quaternary pump (G131A model), using ODS-reversed phase column (C18, 25 \times 0.46 cm i.d. 5 μ m), and a mobile phase of potassium phosphate buffer/methanol 97/3 (v/v) with flow rate 1 ml/min. After sample injection (20 μ l), peaks were visualized at wavelength of 270 nm. Brain serotonin in addition to noradrenaline and dopamine concentrations in different groups were calculated using external standard method using peak areas. Serotonin, noradrenaline, and dopamine standards were prepared in serial dilutions, and each dilution was injected in the HPLC column to determine the peak area of each one. Linear standard curves were made: plotting peak areas versus the corresponding concentrations. Concentrations of brain samples for each parameter were then determined according to its corresponding standard curve.

Determination of brain acetylcholinesterase Activity

Activity of AChE in brain of rats were determined by Ellman et al., (16) using a colorimetric kinetic assay (Biochemical Enterprise Milano, Italy).

Statistical analysis

Data analysis was performed using version 16 of the SPSS (statistical package for the social sciences) program, and Microsoft Excel 2007. Significant difference between values was evaluated using one-way ANOVA and Student's t-test and the value at $p < 0.05$ indicated a statistically significant difference.

RESULTS

The present results, as illustrated in Figure 1, indicated that fasting blood glucose level in STZ-injected rats (6.0 mg/0.5 ml/100 g body weight) exhibited a sharp elevation of a significant change at $p < 0.05$ with a percentage difference of 187.7 % versus control value. In accordance with diabetes

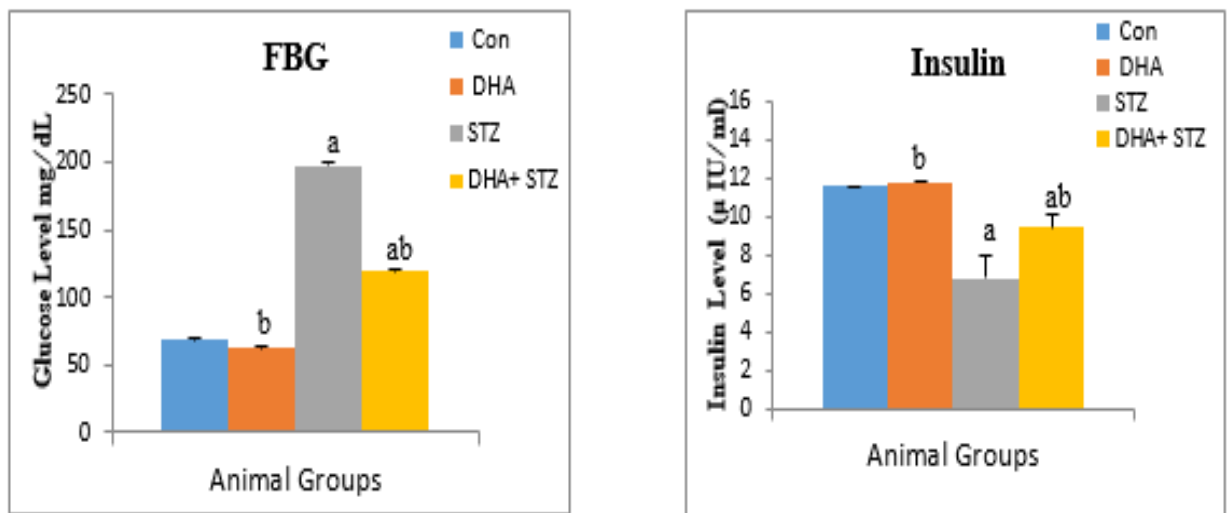


Figure 1. The Effect of docosahexaenoic (DHA) on fasting blood glucose (FBG; mg/dl) and insulin (μ IU/ml) levels in sera of streptozotocin (STZ)-induced diabetic rats. a: significant change at $p < 0.05$ with respect to control group. b: significant change at $p < 0.05$ with respect to STZ-treated group

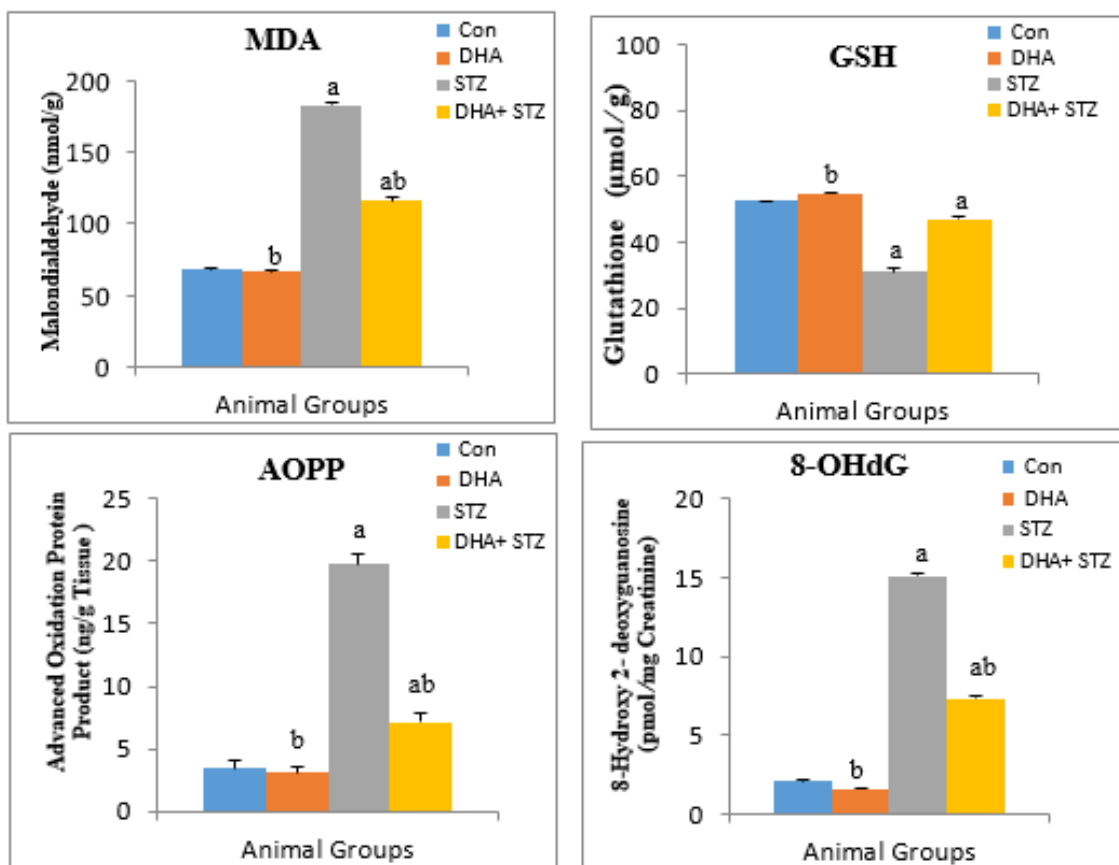


Figure 2. The effect of docosahexaenoic (DHA) on malondialdehyde (MDA), glutathione (GSH) and advanced oxidation protein product (AOPP) contents in the brain and 8- hydroxy-2'-deoxyguanosine (8-OHdG) level in urine of STZ-induced diabetic adult male rats. a: significant change at $p < 0.05$ with respect to control group. b: significant change at $p < 0.05$ with respect to STZ-treated group

induction in rats, insulin level in sera of the same group revealed a significant decrease ($p < 0.05$), with a percentage difference of -41.74 % when compared with the control value, indicating the successful induction of diabetes in the STZ-treated rat group. Meanwhile, DHA alone showed a slight decrease of fasting blood sugar and an increase in insulin level of non-significance compared to control, but were significant when compared to STZ- treated group. DHA + STZ treated rats indicated the potency of DHA in lowering blood glucose and elevating insulin level with significant changes ($p < 0.05$) versus STZ-group values.

As shown in Figure 2, induction of diabetes in the present study was found to provoke a sharp increase in malondialdehyde (MDA), and antioxidant protein product (AOPP) accompanied by a marked decrease in glutathione (GSH) level in the brain of adult male rats, which was significant ($p < 0.05$), with percentage differences of 167.23%, 480.88%, and -40.23%, respectively, when compared to control values. A tremendous increase was also recorded in 8-hydroxy-2'-deoxyguanosine (8-OHdG)

contents in urine, with a significant change $p < 0.05$, with percentage differences of 619.07%, compared to the control group value.

The present results illustrated in Figure 2 indicated that DHA administration to STZ-diabetic rats increased GSH and inhibited markedly MDA, AOPP contents in the brain of rats and also decreased 8-OHdG formation in urine. The observed increase in GSH content and the decrease in MDA, AOPP and 8-OHdG contents were significant ($p < 0.05$) when compared with both STZ-diabetic rat values and control group values, with percentage differences of -10.44%, 69.84%, 108.82%, and 247.62 %, respectively, if compared to control group.

Investigation of the neurotransmitter content in the diabetic rat brain revealed marked elevation in NE, DA and 5-Hydroxytryptamine (5-HT) accompanied with a marked sharp increase in AChE activity as illustrated in Figure 3. These elevations were significant at $p < 0.05$ with percentage differences of 66.47%, 58.06%, 159.32%, and 110.94%, respectively, compared to control values.

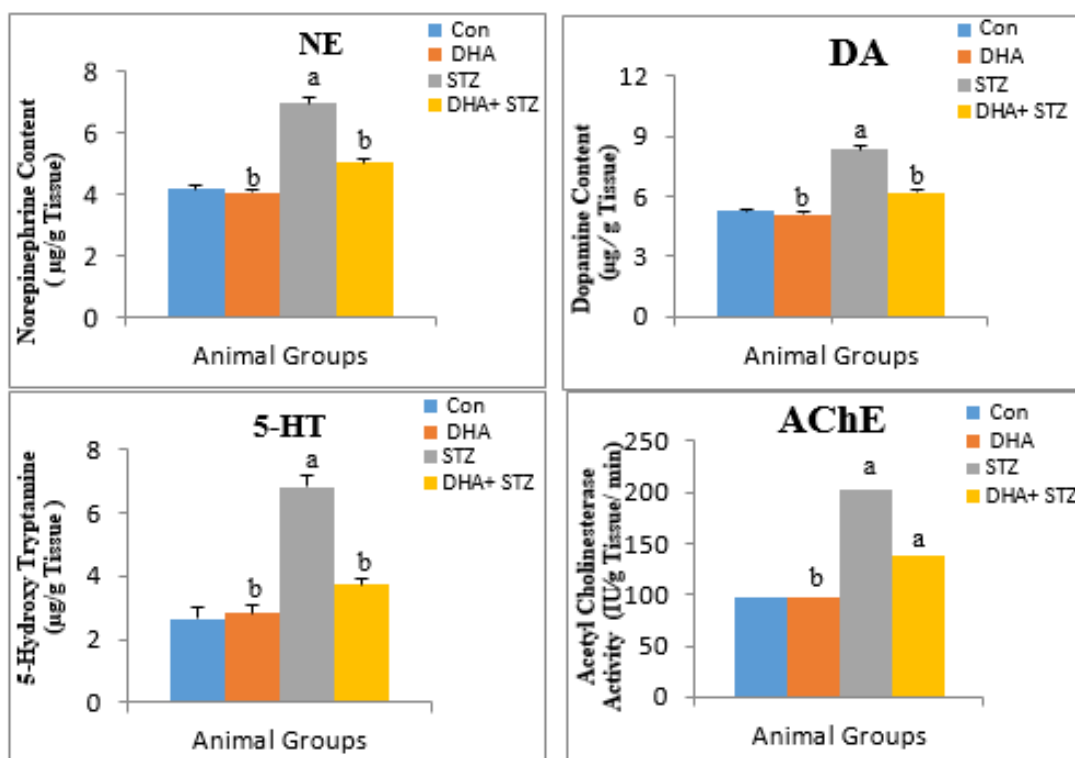


Figure 3. The effect of docosahexaenoic (DHA) on norepinephrine (NE), dopamine (DA), 5- hydroxytryptamine (5-HT) contents and acetyl cholinesterase (AChE) activity in brain of STZ-induced diabetic rats. a: significant change at $p < 0.05$ with respect to control group. b: significant change at $p < 0.05$ with respect to STZ-treated group

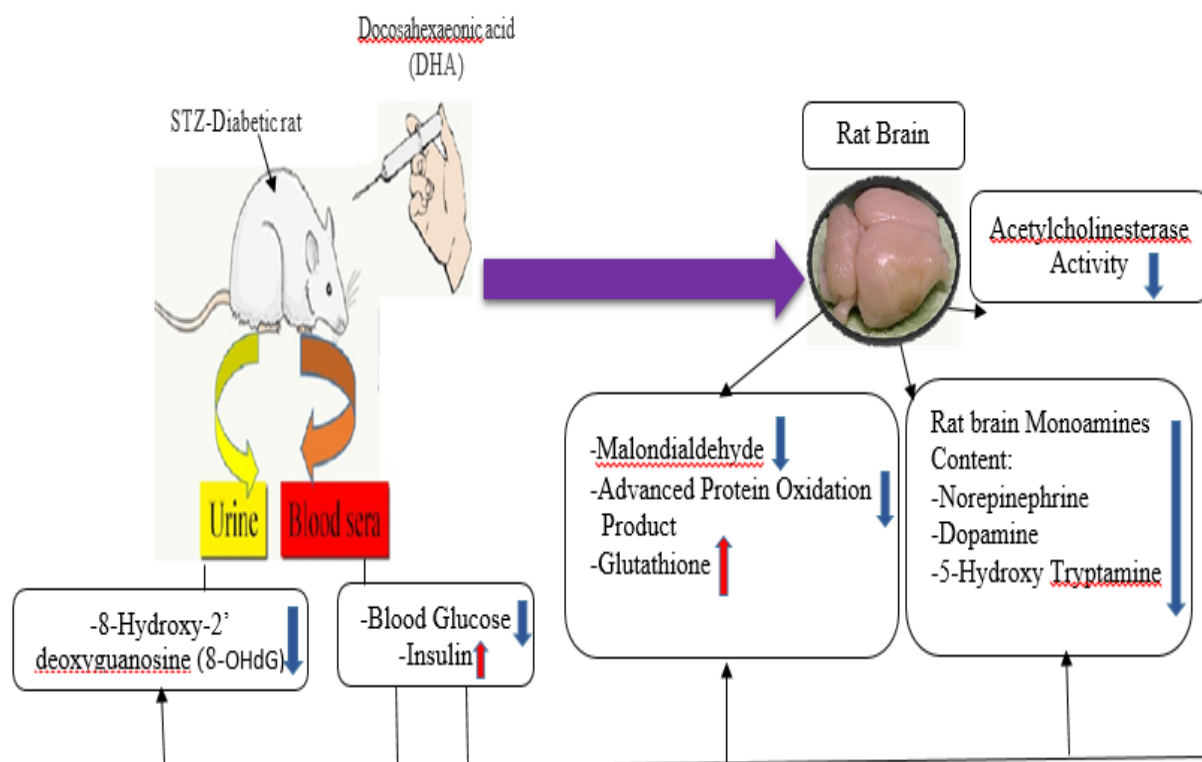


Figure 4. An illustrative graph showing the impact of docosahexaenoic acid on different investigated parameters in STZ-diabetic rats

Meanwhile, DHA administration to male rats caused non-significant changes in their brain monoamines level as it slightly decreased NE and DA, and increased 5-HT level and AChE activity as well, when all compared to control values. DHA + STZ treated rats group revealed DHA ability to decrease the elevated levels of NE, DA, 5-HT contents in their brain as well as AChE activity with a significant change ($p < 0.05$) if compared to STZ- group values and control group, but with percentage differences of 66.47%, 58.06%, 159.32%, and 110.94%, respectively, when compared to control values (Figure 3). An illustrative graphic that shows the impact of DHA acid on different investigated parameters in STZ-induced diabetic rats was presented in Figure 4.

DISCUSSION

In the present study, diabetes mellitus successful induction was reflected by a significant rise in blood glucose levels and low insulin levels, which is in line with El-Yamany et al. (17) and Mohamed et al. (18). DM was reported to be accompanied with altered memory function, cardiac stress, endothelial disturbed function and many complications (1, 19)

could be linked to impaired insulin as insulin signaling affects synaptogenesis and neuronal survival, which leads to memory loss (1).

Data indicated that DHA induced a significant decrease in fasting blood glucose level, which was in accordance with Hussein et al. (20) who documented synergistic effect of nano-encapsulated DHA on DM, together with a significant increase in serum insulin level which could be due to the G-protein-coupled receptor which mediates insulin-sensitizing effects and the anti-inflammatory adipocytes and monocytes/macrophage (21). Moreover, an improved glucose uptake in T2DM could be related to increased residency time of glucose transporters in the plasma membrane. Furthermore, the effect of n-3 PUFA on the adiponectin genes were associated with insulin resistance (2), also inducing glutathione and thioredoxin expression as antioxidant systems in the hippocampus, since DHA acts as an agonist of peroxisome proliferator-activated receptor- γ (22), thus regulating cytokine expression. Also DHA treatment was found to suppress the inflammatory signaling pathways in acute pancreatitis (23).

Inflammation, oxidative stress, and altered glucose metabolism were reported as important

features of both Type II DM and Alzheimer disease (1). Present results indicated significant increases in malondialdehyde and advanced oxidative protein products contents in the brain of STZ-induced diabetic rats accompanied with decreased glutathione level. Diabetic encephalopathy may result as a degenerative brain disease in long-standing diabetic patients. The impact of ROS on neuronal cells in diabetes caused impaired learning and memory, also problem solving, with mental and motor speed problems, especially in type 1 diabetic patients (24). Increased lipid peroxidation was linked to apoptosis under hyperglycemic extremes in the brain, associated with mild cognitive impairment (25). In many pathological conditions, stress within cells triggers mitochondrial oxidative damage resulting in apoptosis and/or necrosis leads to inhibition of neurogenesis (26). Also, DHA was found to increase the levels of antioxidant enzymes as catalase and reduced glutathione levels with increased expression of glutathione peroxidase in the brain hippocampus (27). In addition, it decreased the ROS levels in the cortex and hippocampus of diabetic rats and attenuated the neuronal loss cognition and the locomotor deficits (28). For continuous neurotransmission activity, the brain uses an uninterrupted supply of oxygen (29).

The present study indicated significant elevation in NE, DA, 5-HT levels in accordance with El-Yamany et al. (17). The enhanced level of NE could be attributed to the accumulation of NE resulting from inhibition of presynaptic release of NE and/or increased re-uptake, or a decrease in metabolic degradation of NE. Meanwhile, DA marked elevations may correlate to dysfunction of the brain dopaminergic system, increased D2 receptor density, and increased tyrosine hydroxylase and monoamine oxidase activities. Moreover, 5-HT elevations reflect a reduction in its turnover or functional changes in the diabetic rat brain. As a structural fatty acid in bilayer membranes, DHA was found to concentrate in photoreceptors and in neurons and glia of the nervous system (6). The unique grouping of fatty acids in brain selectively esterifies arachidonic acid and DHA into the sn-2 position of brain phospholipids in neuronal membranes, but not in neuronal cytoplasm (30).

Both choline and long-chained polyunsaturated fatty acids are crucial nutrients for neurodevelopment and mental health during all life time, as they accumulate in tissues for maintenance of

brain cell function (31). The deficient DHA level observed in healthy elderly people suffering from a decline in memory, learning impairment, may lead to increased occurrence of neurodegenerative diseases, as detected in patients with Alzheimer's disease (32). Visual and neurocognitive deficits were also reported in inadequate DHA intake (33). Regulation of the synaptic transmission was reported as a result of a complex metabolic cooperation between three items: the endothelial cell as an energy supply, the astrocyte network for regulation of functional coordination of cells and plasticity, and the presynaptic neuron responsible for the release of neurotransmitters. Modifications of DHA levels inside phospholipid membranes led to glucose transport change (34) and changes in gap junction coupling (35).

Present data indicated that DHA treatment diminished a significant elevation in AChE activity in brain of diabetic rats which evolved from increased substrate level and increased synthesis of the enzyme, due to changes in retrograde axonal transport, which delivers information to the nerve body and thus serves as a protective mechanism for maintaining adequate glucose delivery (18), indicating the ameliorating effect of DHA on AChE activity. Meanwhile, regional DHA signaling was mediated by phospholipase A2, a critical enzyme for muscarinic cholinergic signaling (36, 37), which increases the incorporation of DHA into synaptic membranes and improves signal transduction, enhancing glutamatergic (38) and dopaminergic (39) synaptic activities, as well as noradrenaline release in cultured cells (40).

The membrane-bound enzymes related neurotransmission, signal transduction controlling neurotransmission, and neuronal growth factors are all affected by the fluidity of the cell membrane which was reported to be augmented by DHA, as its deficiency may participate in micro-aggregation formation and conformational changes of receptors/enzymes in the membrane (41). Fluidity of the neuronal membrane is important for receptors to enable them to recognize neurotransmitter-containing vesicles and transmitting messages as its rigidity leads to less competition of neurotransmitters and transmitting signals by the receptors. The neuronal plasma membrane fluidity was correlated with the neurobehavioral effects and avoidance-related memory function (42). An increased size and complexity of the brain tissue after DHA treatment was proposed by Bandarra et al. (43), which could further

lead to increased mental, behavioral and motor skills development due to maintaining the integrity and function of neurons.

NeuroprotectinD-1 (NPD-1; DHA-derivative), a bioactive effector in neuronal tissues may generate cerebral protection during ischemia (44) and its formation was stimulated by increased oxidative stress and inflammatory cytokines through the tandem phospholipase A2-lipoxygenase action on free DHA (6). Also, NPD1 was reported to reduce formation of β -amyloid peptide involved in Alzheimer's disease progression, stimulating anti-apoptotic genes expression while reducing the expression of pro-apoptotic genes, which therefore inhibits apoptosis due to oxidative stress (45). NPD-1, enhanced the production of a disintegrin alpha-secretase, a metallo-protease showing both neurogenic and neurotrophic properties, evidenced by modulated hippocampal function, increasing thus the newly born neurons in dentate gyrus of adult rats by neurogenesis (32).

Results showed a significant lowering effect of DHA on 8-OHdG level in urine of DHA + STZ- rats, minimizing DNA oxidative damage and oxidative stress in tissues, which may be attributed to increased levels of mRNA of the antioxidant molecules or diminished DNA double-strand breaks, which decreased both the γ -H2AX foci formation and ATM activation (a major kinase orchestrating DNA damage response) (29). The results also suggested a DHA genomic protective effect through the up-regulation of nuclear factor-2 thus attenuating oxidative stress-

induced DNA damage. Moreover, DHA was found to influence differentiation of neural stem cells into neurons, both in vitro and in vivo, and suppress the cell death (29).

CONCLUSION

The present study showed that docosahexaenoic acid administration to streptozotocin-injected rats enhanced serum insulin level indicating the antioxidant role of docosahexaenoic acid against streptozotocin action on the pancreas which led to lowering of blood glucose level and reducing 8-hydroxy-2'-deoxyguanosine level in their urine and modulation of brain oxidant/antioxidant status, acetylcholinesterase activity and monoamines of diabetic rats.

Conflict of Interest

The authors declare no conflict of interest.

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Authors contribution

All authors contributed equally in this work

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Dokosaheksaenoična kiselina moduliše oksidativni stres i nivo monoamina u mozgu miševa sa dijabetesom izazvanim streptozotocinom

Sahar Mohamed Mahmoud¹, Yasmin Abdel Latif², Hisham Orban², Amr Mahmoud Ibrahim², Jihan Hussein²

¹Departman za zoologiju, Fakultet nauka, Univerzitet u Kairu, Kairo, Egipat

²Departman za medicinsku biohemiju, Nacionalni istraživački centar, Kairo, Egipat

SAŽETAK

Prevalencija dijabetesa melitusa (DM) raste u mnogim zemljama. Niža prevalencija dijabetesa melitusa tipa 2 i ostalih poremećaja metabolizma šećera primećena je kod populacija koje konzumiraju veće količine n-3 polinezasićenih masnih kiselina, koje se uglavnom nalaze u ribi. Dokosaheksaenoična kiselina (DHA) je važan signalni molekul neophodan za kontinuirano održavanje funkcije mozga od strane centralnog nervnog sistema. Cilj ovog istraživanja bilo je rasvetljavanje uloge DHA u kontroli vrednosti glikemije i modeliranju oksidativnog/antioksidativnog statusa i nivoa transmitera u mozgu pacova sa dijabetesom. Dijabetes je bio izazvan jednom dozom subkutano date injekcije streptozotocina (STZ) (6,0 mg / 0,5 ml /100 g telesne težine). Eksperimentalni pacovi soja Vistar, muškog pola, nasumično su podeljeni u četiri grupe: kontrolnu grupu, DHA grupu, miševe kod kojih je dijabetes izazvan streptozotocinom i STZ+DHA grupu. Svi miševi ubijeni su nakon mesec dana i urađena je kontrola glukoze, nivoa insulina, oksidativnog stresa u mozgu, a takođe su provereni i nivoi monoamina. Davanje DHA značajno je poboljšalo vrednosti glukoze nakon noćnog gladovanja i nivoa insulina u poređenju sa DHA+STZ grupom i smanjilo je nivo 8-hidroksi-2'-deoksivanozina u urinu. Pored toga, tretiranje miševa sa dijabetesom izazvanim streptozotocinom dokosaheksaenoičnom kiselinom pokazalo je smanjenje sadržaja malondialdehida i poboljšanje proizvodnje proteina u toku oksidacije, kao i značajno povećanje sadržaja glutaciona u mozgu pacova tretiranih pomoću DHA+STZ. Takođe, došlo je i do smanjenja nivoa monoamina u mozgu pacova. U zaključku: DHA je modulirala nivoa povećanog oksidativnog stresa i neurotransmitera, kao i aktivnost acetilholinesteraze u mozgu pacova sa dijabetesom, kroz povećanje nivoa insulina u serumu.

Ključne reči: dijabetes, dokosaheksaenoična kiselina, oksidativni stres, neurotransmiteri, insulin