Docosahexaenoic acid reduces oxidative stress and restores antioxidant capacity in discrete regions of the aged rat brain

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ABSTRACT

The present study was designed to assess whether a correlation exists between the degree of lipofuscin accumulation in various regions (hippocampus, cerebral cortex and cerebellum) of the rat brain and the rate of lipid peroxidation/antioxidant status. Twelve male Wistar rats of each group of three, six, twelve and twenty four months were selected. Furthermore each group were divided into two subgroups i.e., normal saline treated control (n=6) and 100 mg/kg bw. docosahexaenoic treated experimental rats. For biochemical assays (lipid peroxidation and antioxidant status), brain regions were dissected after sacrificing rats by an overdose of sodium pentobarbitone. Protein content, protein carbonyl, SOD, catalase, glutathione peroxidase and glutathione reductase were estimated by standard methods. Oxidative stress markers (LPO, LIF and PC) in 3 different brain regions were significantly increased while the activities of antioxidant enzymes (SOD, CAT, GPx and GR) in the same brain regions were significantly depleted. Reduced and oxidized glutathione ratio showed remarkable depletion in the old group in comparison with the same brain regions of young rats. The DHA treated rats significantly reduces the oxidative stress markers when compared with their respective control. Our results suggest that increment of the rate of lipid peroxidation is well correlated with the decline in the antioxidant status of the various brain regions. The morphological indicator of lipids-peroxidation, neuronal lipofuscin, formed consequent to mitochondrial residues remaining after lysosomal degradation, also exhibit regional heterogeneity and linear increment with age, while DHA exhibited as an antioxidant and anti-aging properties.

Key words: Lipofuscin, Lipid peroxidation, Antioxidants, Rat brain, Regional heterogeneity.

INTRODUCTION

Aging is a biological phenomenon concerning all living multicellular organisms and it is thought to be a degenerative process caused by accumulated damage that leads to cellular dysfunction, tissue failure, and death. It is now generally accepted that aging and eventual death of multicellular organisms is to a large extent related to macromolecular damage by mitochondrially produced reactive oxygen species, mostly affecting mechanisms to remove damaged structures completely results in the progressive accumulation of garbage, including cytosolic protein aggregates, defective mitochondria, and intralysosomal indigestible material. The mitochondrial free radical theory of aging has taken centre stage for several decades. According to this theory, reacting oxygen species (ROS) are considered to be unwanted toxic by-products of aerobic metabolism that induce oxidative damage to various cellular macromolecules due to their high chemical reactivity. These free radicals are mainly produced by the mitochondrial respiratory chain as a result of electron transport and the reduction of the oxygen molecule. Toxic effects of ROS on cellular components lead to accumulation of oxidative damage which causes cellular dysfunction with age. The free radical theory has been one of the most popular theories of aging for many years. Scientific research on different model organisms aiming to verify the theory has produced abundant data, supporting the theory or, on the contrary, suggesting strong evidence against it. Accumulation of intracellular granules of the pigment, lipofuscin, with the passage of time has been well-documented in postmitotic cells have ultrastructurally quantitated the age-dependent deposition of aging pigment—called —lipofuscin (LIF) in the rat brain demonstrated that strong linear correlation seems to exist.
between the degree of LIF accumulation and the chronological age. Therefore, LIF is the time tested biomarker of aging and cellular degeneration. The science of anti-aging has come a long way. People who are serious about extending their lives are taking advantage of additional anti-aging supplements to compensate for age related defects. Anti-aging supplementation employs the latest scientific research to slow down condition associated with aging through optimal nutrition and all regeneration. Docosahexaenoic acid (DHA) is the main polyunsaturated fatty acid (PUFA) in the phospholipid fractions of the brain and is essential for normal neuronal function. The maintenance of sufficient concentrations of this PUFA is essential for behavioral, learning and memory. PUFAs supplementation may help to reduce the risk of onset of aging or may reduce the insult to brain functions. Earlier some of the studies reported that DHA may have a protective role against oxidative stress as demonstrated by decreased lipid per oxidation, both ex vivo and in vivo. Fortunately, not much work has been done on the effects of natural products in this thrust area of research moreover, in the search for a new drug which may offer neuroprotection by controlling both behavioral and biochemical we have tested docosahexaenoic acid in different parts of the brain viz hippocampus, cerebral cortex and cerebellum.

MATERIALS AND METHODS

Animals:
Forty Eight male albino rats of four different age groups (3, 6, 12 and 24 months) were taken from Animal house of the University. The animals were separately housed in polypropylene cages at a room temperature, which was maintained at a temperature of 22 ± 2 °C, relative humidity of 50 ± 10 % and 12h light dark cycles. They were fed a commercial pellet diet and allowed access to water ad libitum. The ICMR ‘Guide for care and use of animals’ was followed and the Institutional Animal Ethics Committee approved this study prior to the initiation of the experiment and also approved all experimental protocols.

Behavioral Studies:
After 30 days of treatment rats were allowed to move for behavioral study. Following behavioral parameters were performed with control and experimental rats.

1. **Spontaneous motor activity (SMA):**
   SMA was measured by scored on a scale of 0 – 9 in which SMA in control group will be assigned score 4.

2. **Righting reflex:**
   Rats were placed on their back to see if the animal could quickly right itself and assume a normal posture. Neurological deficits were indicated by an inability to regain normal body posture within five seconds.

3. **Catalepsy:**
   A condition in which body or limbs remain passively in any position in which they can be placed. This test is used by placing forepaws on a metallic rod placed at height of 6 cm and forepaws were not observed withdrawn within 10 sec. catalepsy were considered positive.

4. **Muscle coordination test (Rota rod):**
   The period of stay on rotating rod (speed: 5 rotations / min; Total duration of test 2 min) for each control and experimental rats were recorded by Rotamex (Techno Electronics, India). The rats were trained to stay for period of 2 min on rotating rod and only trained rats were included in the study.

**Preparation of Tissue Homogenate:**
Rats were sacrificed by anesthetic overdose of sodium pentabarbitone (100 mg/kg). The whole brain was removed within one minute and dissected into discrete parts i.e., hippocampus, cerebral cortex and cerebellum. The brain parts were homogenized in 10% w/v cold potassium phosphate buffer (50 mM; pH 7.4). The whole homogenate was divided into two portions; the first portion was centrifuged at 700 x g for 20 min in order to determine lipid peroxide level and GSH level. The second portion of the homogenate was centrifuged at 20,000 Xg for 30 min for the determination of the activity of antioxidant enzyme.

**BIOCHEMICAL ASSAY**

**Protein carbonyl content:**
Protein carbonyl (PC) content was measured by the method of Liu et al. Protein was precipitated with 20% trichloroacetic acid (TCA). After centrifuging at 11,000 x g and 4 °C for 15 min, the supernatant was removed. The pellet was resuspended in 0.5 ml of 10mM 2, 4-dinitrophenylhydrazine (DNPH)/2M HCl (50 °C). Samples were kept in a dark place and vortexed every 10 minutes for one hour. The samples were precipitated with 0.5 ml of 20% TCA, and centrifuged at 11,000 x g and 4 °C for 3 min. The same procedure was repeated with 10% TCA for three times. Precipitate was dissolved in 2ml of NaOH at 37 °C. Absorbance was recorded at 360 nm. Protein carbonyl levels were expressed as nmol carbonyl/mg protein using ε max =22,000M−1 cm−1.

**Lipid peroxidation:**
Lipid peroxide (LPO) was estimated in the brain regions using thiobarbituric acid (TBA) test described by Ohkawa et al. 0.1 ml of homogenate of brain regions was added to the test tube containing 0.2 ml of 8% of SDS, 1.5 ml of 20% aceitic acid solution (pH 3.5) and 1.5 ml of 0.8% TBA solution. The mixture was diluted to 4 ml with distilled water and heated at 95°C for 60 minutes. After cooling on ice, the samples were extracted with 4 ml mixture of n butanol and pyridine (15: 1 v/v). The organic phase was collected and the absorbance measured at a wavelength of 532 nm. The concentration of TBA was determined using the extinction coefficient of 1.56 x 105 M−1 cm−1.

**Lipofuscin:**
Lipofuscin (LIF) was measured using 2:1 chloroform-methanol extraction mixture. The concentration was measured with a fluoro-spectrophotometer at an excitation...
maximum of 360 nm and emission maximum of 420 nm. The content of the fluorescence was determined using quinine fluorescence as a standard. Data were presented as U/g tissue. One unit (U) of lipofuscin is defined as fluorescence of 0.01 g/ml quinine sulfate.

**Superoxide dismutase:**
The superoxide dismutase (SOD; EC 1: 15.1.1.1) activity was determined from its ability to inhibit the reduction of NBT in presence of PMS. The reaction was monitored spectrophotometrically at 560 nm. The SOD activity was expressed as U/mg protein (1 unit is the amount of enzyme that inhibits the reduction of NBT by one half in above reaction mixture).

**Catalase:**
Catalase (CAT, EC 1.11.1.6) activity was assayed using hydrogen peroxide as substrate; the decomposition of H₂O₂ was followed at 240nm on spectrophotometer. The CAT activity was expressed as U/mg protein.

**Glutathione peroxidase:**
The glutathione peroxidase (GSHPx, EC 1.11.1.10) was assayed using GSH, NADPH and H₂O₂ as reactants. The oxidation of GSH into GSSG was measured in terms of oxidation of NADPH to NADP⁺ and assayed as decrease in the absorbance of reaction mixture at 340 nm on spectrophotometer. The activity of GSHPx was expressed as n moles of NADPH oxidized / min / mg protein.

**Reduced glutathione:**
Reduced glutathione was measured in deproteinized supernatant of the brain regions. Tissue homogenate was deproteinated with tetrachloroacetic acid, centrifuged and supernatant was used for the estimation of reduced glutathione (GSH) with the help of Ellman reagent (5, 5'-dithiobis (2-nitro benzoic acid). The optical density of the pale colour was measured on the spectrophotometer on 412 nm. An appropriate standard (pure GSH) was run simultaneously. The level of GSH was expressed as µg / g tissue.

**Histopathological Studies:**
After 30 days of experimental treated and control rats were sacrificed by cervical dislocation. Different brain regions cerebral cortex, hippocampus and cerebellum were removed and rinsed with physiological saline. A portion of the tissue samples were fixed with neutral formalin dehydrated with different concentrations of ethyl alcohol and embedded in paraffin. Tissue sections were cut (4 µm) and stained with hematoxylin and eosin for histological grading.

**Statistical Analysis:**
The data were summarized as Mean±SEM. The significance of mean difference between two groups was evaluated by Student’s t-test. The significance (*) were evaluated using one way ANOVA.

**RESULTS AND DISCUSSION**
Brain is a heterogeneous conglomeration of many discrete "little organs", rather than one large organ. Cerebral cortex itself exhibits around 52 distinct Brodmann’s areas! Hence, instead of evaluating neurochemical parameters in the whole brain or its 3 major components (hippocampus, cerebrum and cerebellum), as commonly observed in the literature, we have dissected out hippocampus, cerebral cortex and cerebellum of both control and DHA treated rats for the estimation of oxidative stress parameter and histopathological changes followed by behavioral study. To date, only very few studies have raised the question of how life-long aging could affect later performance in different learning tasks. In the present study, we evaluated non-invasive method of behavioral changes in control and DHA treated young and aged rats. The body weight of the control and experimental rats were observed during the experimental period from 0 days, 15 days and 30 days. Body weights were found to be gradually increased in control and experimental rats. There were insignificant (p>0.05) change between control and experimental rats (figure-1).
muscle in-coordination were found to be significantly ($p<0.01$) increased in the 24 old months and 12 months old experimental rats when compared with their age matched control rats. It has been reported that brain learns to relax some muscles like the front of our shoulders and hamstrings and therefore, postural development obviously depends on muscular development as well as on neural control.\(^2\) Muscles which are important for maintaining a stable posture involve trunk muscles as well as so called postural muscles in the extremities. Posture position and catalepsy were found to be significantly changed when compared with the controls while it was reverses in DHA treatment. Significant altered posture position in aged rats when compared with the DHA treated 24 months old rats.

Cerebellum. Increased activation of GABA receptors impairs motor coordination.\(^27\) In the present study, reduced muscle in-coordination ability was observed in the 24 month old rats. It is suggestive that aging may induce muscle weakness and defects in neural incarnation. The obtained results are in agreement with the findings of previous study.\(^28\) It is suggestive that loss of brain and cerebellum may be one of the causes of neuromuscular deformities. Furthermore, we observed that co-administration of DHA to 24 months rats significantly reversed their behavioral changes. With the passage of time, either generation of ROS is increased, or the ability to detoxify ROS is compromised. Although decreases in the levels or activity of the antioxidant enzymes, superoxide dismutase (SOD) and catalase, may be a contributing factor in increased ROS and subsequent cellular damage, compromised mitochondrial function, membrane permeability and/or increased metal content are additional factors that may potentially lead to increased generation of ROS. The mitochondria contains array of respiratory chain, where water is produced from the reduction of two molecules of oxygen and produced energy in the form of ATP.\(^29\) Defects in the respiratory chain yield partially reduced free-radical products of oxygen, such as superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$) and $OH^-$ radicals, which are highly toxic and damage normal cellular constituents by reacting with them\(^30\) and hence they are called reactive oxygen species (ROS). Aging has been implicated in human neurodegenerative diseases like Alzheimer’s. Possible mechanisms of aging been related to cell damage via free radical production. Increased lipid peroxidation and protein oxidation are the major consequence associated with oxidative stress. Brain is highly enriched in long chain polyunsaturated fatty acids (PUFAs) particularly docosahexaenoic acid (DHA) which play important role in brain structural and biological functions. Transport of long chain PUFAs from plasma may important roles because of the limited ability of brain to synthesize long chain PUFAs, in the face of high demand for them. Although, several proteins involved in facilitated fatty acid transport (e.g. Fatty acid transport protein, fatty acid binding protein and very long chain acyl-coenzyme a synthetase) have been found in brain.\(^31\)\(^32\)

Effect of DHA on oxidative stress parameters: In the present study, effect of DHA on oxidative stress parameter namely LPO, PC, LIF, SOD, CAT, GPx and GSH are presented in the table-1. The lipid peroxide levels were found to be significantly ($p<0.01$) reduced in the hippocampus, cerebral cortex and cerebellum of the 24 months old rats following DHA treatment when compared with the aged matched control rats. The concentration of protein carbonyl content were found to be significantly ($p<0.01$) reduced following DHA co-administration in hippocampus, cerebral cortex and cerebellum of the 24 months old rats and hippocampus of 12 month old experimental rats when compared with their age matched control rats. The lipofuscin contents were found to be significantly ($p<0.01$) reduced in the hippocampus,

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**Figure 2.** The SMA, Catalepsy, gait and rota rod score of the control and experimental rats are expressed as mean ± SEM for six animals of each group. The gait of the control and experimental groups analyzed by One way ANOVA followed by Student Newman–Kuels test. The significant comparison represents between control (CT) and experimental (Exp) rats (*). The minimum P value $<0.05$ was considered significant.

Previously it has been reported that increased exposure of neurotoxicant reduces motor ability and posture of the body.\(^23\) Catalepsy and gait is the position that the animals are unable to correct externally imposed postures. There is pharmacological evidence for stimulation of dopaminergic neurons by noradrenergic neurons in the brain.\(^24\) In the present study, increased catalepsy was observed during the progression of aging to 24 months old rats. It has been reported that, catalepsy is a symptom of certain nervous disorders or conditions such as Parkinson’s disease and epilepsy.\(^25\) Moreover, protein kinase-C has been suggested as a mediator of cataleptic behavior in aged rat brain.\(^26\) The rota rod performance test is a performance test based on a rotating rod with forced motor activity being applied, usually by a rodent. The test measures parameters such as riding time (seconds) or endurance. Some of the functions of the test include evaluating balance and coordination of the subjects; especially in testing the effect of experimental drugs. Motor coordination is mainly modulated in cerebellum. A main player in modulation of motor coordination is GABAergic neurotransmission in...
Table 1. The lipid peroxide levels (LPO; nmole MDA /g tissue), Protein carbonyl (PC; nmole carbonyl content/mg protein), lipofuscin (LIF; unit / g tissue) superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSHPx) are expressed in unit / mg protein, while, reduced glutathione (GSH) is expressed in µg / g tissue. The result expressed as Mean ± SEM in six rat of each group. The significant comparison represent between control (CT) and experimental (Exp) rats (*). The minimum P value<0.05 was considered significant.

<table>
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<td>319.3±30.5</td>
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cerebral cortex and cerebellum of the 24 months and 12 months old experimental rats following DHA treatment when compared with the aged matched control rats. Following DHA co-administration, activity of superoxide dismutase were found to be significantly \((p<0.01)\) increased in hippocampus, cerebral cortex and cerebellum 24 months old rats and hippocampus and cerebral cortex of 12 months old rats when compared with their age matched control rats. DHA co-administration, activity of catalase were found to be significantly \((p<0.01)\) increased in hippocampus, cerebral cortex and cerebellum of the 24 months and cerebral cortex of 12 months old experimental rats when compared with their age matched control rats. DHA co-administration, activity of glutathione peroxidase were found to be significantly \((p<0.01)\) increased in hippocampus, cerebral cortex and cerebellum 24 months old rats and cerebral cortex of 12 month old experimental rats when compared with their age matched control rats. DHA co-administration, activity of glutathione levels were found to be significantly \((p<0.01)\) increased in hippocampus, cerebral cortex and cerebellum of the 24 old experimental rats when compared with their age matched control rats. In the present study, rapid increase of lipid peroxides, and lipofuscinogenesis are closely related to peroxidation of lipids. Our result demonstrating increased LPO and their peroxidative products in 12 months and 24 months old rats supported to the findings of Newairy et al. The age-related changes in the brain lipids and protein, caused by reactive oxygen species (ROS), were evaluated by estimating lipid peroxide level and protein oxidation. Their mean levels in the selected brain regions of the old rats were significantly higher in comparison with the young ones. However, when mean levels of LPO and PC markers in the selected regions of the 24 months DHA treated rats were compared with the values of age matched control groups. Accumulation of lipofuscin may be the result of an imbalance between formation and removal mechanisms. Lipofuscin is often considered a hallmark of aging, showing an accumulation rate that inversely correlates with longevity. Aside from large lipid content, lipofuscin is known to contain sugars and metals, mercury, aluminum, iron, copper and zinc. In the aging human brain, deposits of lipofuscin are not uniformly distributed but are concentrated in specific regions of functional interest. Robust evidence suggests that lipofuscin is not benign but can distort and displace the endoplasmic reticulum, the protein building machinery of the cell. As evident by our study we reported here increased accumulation of lipofuscin in 24 months old rats is the marker of aging. The decrease in the antioxidative defenses (SOD, CAT, GSH-Px, GR and GSH) accompanied with the increased levels of lipid peroxides. On the other hand, co administration with DHA found to be reduced LPO levels in different regions of the brain. Among various antioxidative mechanisms in the body, SOD is thought to be one of the major enzymes which protects against tissue damage caused by the potentially cytotoxic free radicals. It is therefore possible that the decrease in SOD activity with age may be closely related to the mitochondrial stress. The decrease in SOD activity may further accelerate the process of neurodegeneration. Furthermore, some authors even suggest a causal relationship between activities of antioxidant enzymes and the life span of animal species. It is point out that in general the activity of catalase declines during the maturation of the animal to adulthood. It is also reported a marked and progressive decline in catalase activity in all of the brain regions studied our results demonstrating aging mediated decrease in SOD and CAT activities both are in concordance with the findings of earlier workers. During the present investigation, specific activity of GPx was found to be highly decreased in hippocampus, cortex and cerebellum old rats as compared with the age matched DHA treated rats. In the present study, histopathological changes in the cerebral cortex, hippocampus and cerebellum of 24 months old and control rats were investigated and presented in the figure -3. In the histopathology picture of cerebral cortex of control (figure-3A) rats exhibited disorganization of cells in the successive layers was seen. Layering arrangement of the cells was quiet disrupted. Cells appeared to be larger in size and large vascular spaces were seen around them (figure-3B) while, the age matched DHA treated rats showed less number of changes as compared to their respective controls. Histology of hippocampal section showed revealed neuronal cell with pykned cell bodies, chromatim condensation in general and apparent flame-shaped appearance of cells in the hippocampus of 24 months old control rats (figure-3C). The DHA administrated months 24 old rats exhibiting Nissil granules content in pyramidal cells cytoplasm and with well sealed architecture of the neuron (figure-3D). Photomicrographs of the H&E stained section of the cerebellum of the 24 month old rats revealed enough to disrupt the normal arrangement of three layers. Large spaces in between Purkinje’s cell layer and molecular layer or granular layer were seen (figure-3E). The section obtained from age matched DHA treated rats (figure-3F) exhibited well maintained architecture and no apparent degenerative changes in any of the three layers when compare to their age matched control rats.

Figure-3. Light photomicrograph of H&E stained section of 24 months old rats. Cerebral cortex control (A) and DHA treated (B); hippocampus of control (C) and DHA treated (D); cerebellum control (E) and DHA treated (F) at (100x).
The supplementation of DHA found to be incomparable changes were observed in aged rats with controls. Our results show protection against aging by DHA involving the damage of lipid and protein layer of the cell by the production increased rats of reacting oxygen species. It has been shown that DHA exhibited protective effect against neuronal aging. We propose that DHA protect against cognitive impairment in aging by increasing the formation of neuronal membrane, specifically synaptic membrane and dendritic spines and thereby promote neurotransmission. It is reported that DHA administration increases brain phosphatide levels, and the density of hippocampal dendritic spines, which require additional synaptic membrane. DHA administration reversed the decline in GluR2 glutamate receptor subunits, thereby improving glutamatergic transmission in the hippocampus. The supplementation of DHA found to be incomparable changes were observed in control and experimental rats. Our results show protection against cellular aging by DHA involving the damage of lipid and protein layer of the cell by the increased rats of reacting oxygen species. It has been shown that DHA exhibited protective effect against metal. As we know, brain tissue contains large amounts of polyunsaturated fatty acids, which play important role in brain structural and biological functions. Transport of long chain PUFAs from plasma may important roles because of the limited ability of brain to synthesize long chain PUFAs, in the face of high demand for them. Several hypotheses have been proposed to describe the role of DHA in the brain, which conferred by lipid-bound DHA in the membrane bilayer, when DHA is in short supply, the structural and functional integrity of the nerve cell is compromised. In the present study, DHA co-administration reduces the LPO and PC and restore the capacity of antioxidant in particular aged rat brain regions. The quantitatively estimated LIF in the different brain regions exhibited reduced lipofuscinogenesis. It is suggestive that the DHA supplementation may protect from neuronal aging either by compensatory mechanism to restore membrane lipid or by reducing oxidative stress. Therefore, present study reflects linear correlation between lipofuscinogenesis and advancing age while DHA modulating these changes.

CONCLUSION

In conclusion, physiological aging directly correlates with behavioural, biochemical and cellular changes in different parts of the rat central nervous system. Hippocampus was more affected regions of the brain, it is evident that a close relationship exist between aging and oxidative stress, structural changes and behavioural changes. DHA have potential applications in neuromodulation. The 100mg/kg DHA Co-administration shows neuroprotective against neuronal aging. DHA contain Antioxidative action, free radical scavenging capacity as well antiaging as it reduces lipid peroxidation, protein oxidation, and lipofuscinogenesis as well as restore cognitive ability. However, DHA additionally, also significantly recovered endogenous antioxidants (i.e., CAT, SOD, GPx, GR and GSH) which protect neurons against ROS. Hence it is recommended that DHA has potential for further development as a therapeutic agent for aging and other neurodegenerative diseases.

REFERENCES


