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Antioxidant Defence of Dietary Squalene Supplementation on n-3 Poly Unsaturated Fatty Acids (PUFA)-Mediated Oxidative Stress in Young and Aged Rats

T. Obulesu[1, 2], K. K. Asha[1], R. Anandan[1]*, S. Mathew[1], B. Ganesan[1], G. Krishna[2] and W. S. Lakra[2]

[1] *Central Institute of Fisheries Technology, P. O. Mastyapuri, Cochin - 682 029, India*

[2] *Central Institute of Fisheries Education, Off Yari Road, Versova, Mumbai - 400 061, India*

Abstract

Oxidative stress-induced aberrations in fatty acid composition of central nervous system during progression of aging are known to alter homeostatic processes of the whole organism. Antioxidants that accumulate in brain and neural tissue are potential candidates for prevention or treatment of disorders involving oxidative damage during disease progression. In the present study, an attempt was made to examine the antioxidant effect of squalene against n-3 polyunsaturated fatty acids (PUFA)-mediated oxidative stress in young and aged rats with respect to the changes in lipid peroxidation, enzymatic and non-enzymatic antioxidant status. Dietary co-intake of squalene significantly attenuated n-3 PUFA mediated oxidative stress in different regions of brain tissue of young and aged rats and maintained the cerebral antioxidant status at near normalcy. Though *per se* dietary supplementation of n-3 PUFA significantly elevated the enzymic and non-enzymic antioxidant levels in young rats, it diminished the antioxidant profiles in aged brain striatum, cortex and hippocampus regions. The results of the present study indicated that the combined dietary supplementation of squalene and n-3 PUFA might be a viable therapeutic strategy to ameliorate age-associated oxidative stress in brain tissue.

Keywords: Aging, oxidative stress, central nervous system, squalene, n-3 PUFA

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* *E-mail: kranandan@email.com*

Introduction

Aging is a physiological progression reflected by an increase in the susceptibility to neurodegenerative diseases. Oxidative stress plays a predominant role in the pathogenesis of aging process and aging related neurodegenerative diseases (Harman, 1992). Throughout life, brain is exposed to oxidative stress and a number of diseases of the brain have been hypothesized to involve free radical-induced oxidative damage (Aravizhagan et al., 2002). Different brain regions like cortex, striatum and hippocampus are highly enriched in non-heme iron, which is catalytically involved in the production of oxygen free radicals (Subbarao et al., 1990). Oxidative stress favours lipid peroxidation in cell membranes of which PUFA are primary targets due to their unsaturation (Ledesma et al., 2012). Cell plasma membranes are prone to lipid modifications caused by free radicals. When an imbalance between reactive oxygen species (ROS) generation and antioxidant formation occurs, oxidative damage to cell targets (lipids, nucleic acids and proteins) can be aggravated (Halliwell et al., 1995). The development of innate drugs based on aging pathophysiology is a current area of study and research.

Highly lipophilic antioxidants that accumulate in neuronal tissue may be potential candidates that can be used for prevention or treatment of age associated neurodegenerative disorders. Squalene possesses antilipidemic, antioxidant and membrane stabilizing properties (Quereshi et al., 1996; Ko et al., 2002; Ivashkevich et al., 1981). Buddhan et al. (2007) reported that dietary supplementation of squalene improved hepatic mitochondrial function during aging in which reactive oxygen species were a major cause. O'Sullivan et al. (2002) reported that squalene was more effective than eicosapentaenoic acid (EPA)

and docosahexaenoic acid (DHA) in protection against H_2O_2 - induced sister chromatid exchange (SCE) in Chinese hamster V79 cells. Omega-3 fatty acids co-administered with statins have synergistic and additive effects on plasma lipids (Vrablik et al., 2009). The elevated intake of unsaturated fatty acids (MUFA and PUFA) and antioxidants could act synergistically to improve cognitive performance (Solfrizzi et al., 2008). Based on the above back ground, an attempt was made to investigate the levels of lipid peroxidation, glutathione (GSH), glutathione dependent enzymes [glutathione peroxidase, (GPx) and glutathione-*S*-transferase (GST)] and antiperoxidative enzymes [superoxide dismutase (SOD) and catalase (CAT)] in brain tissue of young and aged rats induced with n-3 PUFA (extracted from sardine oil) - mediated oxidative stress and treated with dietary squalene (prepared from shark liver oil).

Materials and Methods

Epinephrine, tetraethoxy propane and cholesterol were purchased from M/s. Sigma Chemical Company, St. Louis. MO, USA and used in the study. Squalene (specific gravity: 0.853; refractive index: 1.493; saponification value: 30; iodine value: 344; boiling point: 240-245°C) was prepared from shark liver oil of *Centrophorus* sp. caught in the Andaman waters (Farvin et al., 2004) and PUFA concentrate (Table 1) extracted from sardine oil was used for the experiment. All other chemicals of analytical grade were procured locally.

Table 1. Fatty acid composition of PUFA concentrate prepared from fish oil

Fatty acids	Percentage
C16:0	5.74
C18:0	2.91
C16:1 n-7	3.58
C18:1 n-9	5.52
C20:4 n-6	9.74
C20:5 n-3	27.5
C22:6 n-3	38.2
Others	6.81

Animals and treatement

Male Wistar strain albino rats, weighing 120-150 g [young rats of 2-3 months old (mean age: 78.5 ± 6.42 days)] and 350-400 g [aged rats of 20-25 months old (mean age: 697 ± 47.3 days)] were selected for the study. The animals were housed individually in polypropylene cages under hygienic and standard environmental conditions (28 ± 2°C, humidity 60-70%, 12 h light/dark cycle). The animals were allowed a standard diet [M/s Sai Foods, Bangalore, India] and water *ad libitum.* The research work was carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC).

Investigation protocol

Seven days after acclimatization, the animals were divided into two major groups: Group I consisted of 24 young rats and Group II consisted of 24 aged rats. Each group was further sub-divided into four groups (6 rats each): one control group (Group Ia and Group IIa) and three experimental groups based on the dietary supplementation (at 1% level) of PUFA concentrate (Group Ib and Group IIb), squalene (Group Ic and Group IIc) and squalene + PUFA concentrate (Group Id and Group IId) along with feed for 45 days. On completion of 45 days of supplementation, the animals were sacrificed and the brain tissues were excised immediately and immersed in ice-cold physiological saline and blotted with filter paper. Brain regions (cortex, striatum and hippocampus) were separated by the method of Glowinski & Iversen (1966).

Biochemical assays

GSH was estimated by the method of Ellman et al. (1959). GPx was assayed by the method of Paglia & Valentine (1967). GST was assayed by the method of Habig et al. (1974). SOD was assayed by the method of Misra & Fridovich (1972). CAT was assayed by the method of Takahara et al. (1960). LPO was assayed by the method of Ohkawa et al. (1979) in which the malonaldehyde (MDA) released served as the index of oxidative stress 1,1,3,3-Tetra ethoxy propane malonoldialdehyde bis (diethyl acetate) was used as standard.

Statistical analysis

Analyses were repeated three times, and the results are presented as mean ± standard deviation for six samples. Multiple comparisions of the significant ANOVA were performed by Duncan's multiple

comparisions test. A p value <0.05 was considered as statistically significant. All data was analyzed with the help of statistical package program SPSS 10.0 for windows.

Results and Discussion

Oxidative stress is considered to be a risk factor contributing to age-related increase in oxidized lipids and susceptible biomolecules in the central nervous system during aging that ultimately results in cellular deterioration of brain tissue. Significantly low levels of GSH (Table 2), GPx and GST (Table 3), SOD and CAT (Table 4) and rise in the level of LPO (Table 2) was observed in Group IIa aged rats as compared with Group Ia young rats. Lipid peroxidation *in vivo* has been identified as one of the basic deteriorative reaction in cellular mechanisms of brain aging (Montine et al., 2002). The generation of reactive oxygen species and consequential oxidative stress has been implicated in the mechanism of brain dysfunction due to age-associated neurodegenerative diseases (Schipper, 2004). It is noticed that n-3 PUFA supplementation aggravated the age-associated oxidative stress in Group IIb aged rats as compared to Group IIa aged rats, indicating the PUFA-induced decline of non-enzymatic and enzymatic antioxidants. However, dietary squalene supplementation significantly attenuated n-3 PUFA-

Table 2. Antioxidant effect of dietary squalene on n-3 polyunsaturated fatty acids (PUFA) concentrate mediated oxidative stress in different brain regions of young and aged rats

Parameters	Young				Aged			
	Ia (control)	Ib (PUFA)	Ic (Squalene)	Id (Squalene + PUFA)	IIa (control)	IIb (PUFA)	IIc (Squalene)	IId (Squalene + PUFA)
Lipid Peroxidation								
Cortex	0.96±0.06 ^{a}	1.12±0.08^{a,b}	0.90±0.07^{a}	0.99±0.07 ^{a}	1.34±0.12 ^{b,c}	1.82±0.18^{d}	1.28±0.15^{b,c}	1.45±0.17^{c}
Striatum	0.79±0.05^{a,c}	0.95±0.04^{b}	0.72±0.06 ^{a}	0.84±0.07^{c}	1.23±0.08^{d}	1.59±0.07e	1.18±0.04d	1.28±0.06d
Hippocampus	0.64±0.03 ^{a}	0.86±0.05^{b}	0.57±0.02^{a}	0.72±0.04^{c}	0.84±0.05^{b}	1.12±0.07^{d}	0.75±0.03^{c}	0.91±0.03^{b}
Reduced glutathione (GSH)								
Cortex	0.65±0.02^{a}	0.87±0.03^{b}	0.76±0.02^{c}	0.67±0.03^{a,d}	0.79±0.01^{c}	0.47±0.01^{e}	0.71±0.02^{d}	0.70±0.05^{d}
Striatum	0.82±0.03^{a}	1.15±0.02^{b}	0.98±0.05^{c}	0.79±0.02^{a}	0.94±0.04^{c}	0.41±0.01^{d}	0.67±0.03^{e}	0.80±0.04^{a}
Hippocampus	0.55±0.01^{a}	0.71±0.01^{b}	0.63±0.02^{c}	0.58±0.01^{d}	0.67±0.03^{e}	0.34±0.01^{f}	0.50±0.01^{g}	0.60±0.01^{d}

Results are mean ± SD for 6 animals; one way ANOVA; Duncan's multiple comparision test. Values that have a different superscript (a,b,c,d,e,f and g) differ significantly ($p<0.05$) with each other. Values expressed: GSH, μmol (g wet tissue)$^{-1}$; LPO (lipid peroxidation), nmol MDA release mg^{-1} protein

Table 3. Antioxidant effect of dietary squalene and PUFA on GPx and GST enzyme levels in different brain regions of young and aged rats

Parameters	Young				Aged			
	Ia (control)	Ib (PUFA)	Ic (Squalene)	Id (Squalene + PUFA)	IIa (control)	IIb (PUFA)	IIc (Squalene)	IId (Squalene + PUFA)
Glutathione Peroxidase (GPx)								
Cortex	1.31±0.02^{a}	1.99±0.01^{b}	1.65±0.03^{c}	1.40±0.06^{d}	0.80±0.01^{e}	0.47±0.01^{f}	1.07±0.05^{g}	1.25±0.01^{h}
Striatum	1.84±0.05^{a}	2.64±0.11^{b}	2.24±0.11^{c}	1.90±0.07^{a}	1.42±0.02^{d}	0.75±0.03^{e}	1.52±0.05^{d}	1.78±0.03^{a}
Hippocampus	1.15±0.02^{a}	1.98±0.02^{b}	1.56±0.01^{c}	1.10±0.05^{d}	0.92±0.01^{e}	0.65±0.03^{f}	0.78±0.02^{g}	1.22±0.01^{h}
Glutathione-S-transferase (GST)								
Cortex	7.37±0.25^{a}	9.45±0.33^{b}	8.41±0.31^{c}	7.85±0.26 ^{d}	8.45±0.14^{c}	4.30±0.14^{c}	6.37±0.18^{f}	7.32±0.36^{a}
Striatum	6.60±0.25^{a}	8.21±0.20^{b}	7.40±0.32^{c}	6.30±0.07^{a,d}	8.98±0.31^{e}	5.89±0.19^{f}	7.43±0.24^{c}	6.58±0.26^{a}
Hippocampus	4.79±0.12^{a,d}	6.79±0.14^{b}	5.79±0.18^{c}	5.03±0.16^{d}	7.62±0.19^{e}	3.75±0.05^{f}	5.68±0.16^{c}	4.73±0.23^{a}

Results are mean ± SD for 6 animals; one way ANOVA; Duncan's multiple comparision test. Values that have a different superscript (a,b,c,d,e,f,g,h) differ significantly ($p<0.05$) with each other. Values expressed: GPx, nmol GSH oxidized min^{-1} (mg protein^{-1}); GST, μmol 1-chloro2, 4 dinitro benzene conjugate formed min^{-1} (mg protein^{-1})

Table 4. Antioxidant effect of dietary squalene and PUFA on antiperoxidation enzymes (SOD and CAT) levels in different regions brain regions of young and aged rats

Parameters	Young				Aged			
	Ia (control)	Ib (PUFA)	Ic (Squalene)	Id (Squalene + PUFA)	IIa (control)	IIb (PUFA)	IIc (Squalene)	IId (Squalene+ PUFA)
Superoxide dismutase (SOD)								
Cortex	$0.67\pm0.02^{a,d}$	0.91 ± 0.04^{b}	0.79 ± 0.01^{c}	0.70 ± 0.02^{d}	0.49 ± 0.01^{e}	0.31 ± 0.01^{f}	0.40 ± 0.01^{g}	0.65 ± 0.02^{a}
Striatum	0.85 ± 0.02^{a}	1.58 ± 0.07^{b}	1.21 ± 0.02^{c}	$0.82\pm0.009^{a,d}$	0.78 ± 0.01^{d}	0.54 ± 0.01^{e}	0.66 ± 0.01^{f}	$0.83\pm0.03^{a,d}$
Hippocampus	$0.76\pm0.02^{a,d}$	0.98 ± 0.02^{b}	0.87 ± 0.02^{c}	0.79 ± 0.03^{d}	0.56 ± 0.01^{e}	0.39 ± 0.07^{f}	0.47 ± 0.01^{g}	0.73 ± 0.01^{a}
Catalase (CAT)								
Cortex2	$1.95\pm0.04^{a,d}$	2.96 ± 0.07^{b}	2.45 ± 0.05^{c}	1.99 ± 0.09^{d}	1.87 ± 0.02^{a}	0.82 ± 0.01^{f}	1.39 ± 0.01^{f}	1.99 ± 0.09^{d}
Striatum	$1.73\pm0.02^{a,d}$	3.61 ± 0.03^{b}	2.67 ± 0.04^{c}	1.77 ± 0.08^{d}	1.61 ± 0.01^{e}	0.90 ± 0.01^{f}	1.25 ± 0.04^{g}	1.69 ± 0.02^{a}
Hippocampus	1.63 ± 0.02^{a}	2.41 ± 0.04^{b}	2.02 ± 0.03^{c}	1.68 ± 0.05^{d}	1.04 ± 0.01^{e}	0.82 ± 0.01^{f}	0.93 ± 0.01^{g}	1.56 ± 0.02^{h}

Results are mean ± SD for 6 animals; one way ANOVA; Duncan's multiple comparision test. Values that have a different superscript (a,b,c,d,e,f,g) differ significantly ($p<0.05$) with each other. Values expressed: SOD, one unit of the SOD activity is the amount of protein required to give 50% inhibition of epinephrine autooxidation; CAT, nmol H_2O_2 decomposed min^{-1} mg^{-1} protein

mediated free radical generation in the brain tissue of Group IId aged rats and maintained the levels of enzymatic and non-enzymic antioxidant status at levels comparable to Group Ia young controls. The synergistic action of dietary squalene is conceivably related to the attenuation of n-3 PUFA mediated oxidative stress at neuronal membrane. The isoprenoid moiety present in the squalene is directly involved in the scavenging of reactive oxygen species (Farvin et al., 2006).

Antioxidant therapies may ease the incidence of certain age-related neurological disorders including macular degeneration and dementia. Squalene has long been recognized to possess antioxidant, anti-inflammatory, anticarcinogenic, platelet aggregation inhibiting and metal chelating properties (Farvin et al., 2009). A phase I trial in adult males, given 860 mg of squalene daily for 20 weeks to study the cholesterol-lowering effect of squalene showed that oral squalene is safe and tolerable. Prior reports (Socci et al., 1995) have also shown that long-term dietary supplementation of lipophilic antioxidants improved cognitive presentation in aged rats. Studies by Floyd & Hensley, (2000) have shown that administration of free radical scavengers to senescent animals reverses protein oxidation in rat brain. Of particular relevance to this present study, is a recent study that has revealed that squalene, both endogenous and exogenous, may help to neutralize free radicals before they can exert their destructive activity (O' Sullivan et al., 2002). Squalene is highly lipophilic and, when administered exogenously, it can readily pass across the cellular and subcellular membranes (Kamimura et al., 1989). The ability of squalene to diffuse into intracellular compartments helps in the capabilities of this isoprenoid as a potent antioxidant (Kohno et al., 1995).

The results of the present study indicate that dietary supplementation of squalene is very effective in minimizing age-associated oxidative stress in the brain tissue of aged rats, thereby justifying its use as an antioxidant agent. In conclusion, the antiaging action of dietary supplementation of squalene on neuronal antioxidant defense system is conceivably related to a counteraction of free radicals, or to a strengthening of neuronal membrane by membrane stabilizing action, or to normal maintenance of the activities of free radical enzymes and the level of GSH, which protect neuronal membrane against oxidative damage by decreasing lipid peroxidation.

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