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Salubrious Effects of Dietary Supplementation of Squalene and n-3 Polyunsaturated Fatty Acid Concentrate on Mitochondrial Function in Young and Aged Rats

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Abstract

The present study was designed to investigate the salubrious effects of dietary supplementation of squalene and n-3 polyunsaturated fatty acid concentrate on mitochondrial function in liver tissue of young and aged rats. The combined dietary supplementation of squalene and polyunsaturated fatty acids significantly attenuated age-associated inhibition on the activities of mitochondrial tricarcarboxylic acid (TCA) cycle enzymes (isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase) and respiratory marker enzyme (NADH dehydrogenase) and maintained the hepatic energy status at near normal. Co-supplementation of squalene and n-3 PUFA exerted synergistic effect in counteracting the reactive oxygen species in the liver mitochondria of aged rats by maintaining the mitochondrial antioxidant status at level comparable to that of young controls. The results of the present investigation have shown that the combined dietary intake of squalene and PUFA concentrate is more effective in ameliorating the age-associated aberrations as compared to *per se* supplementation.

Keywords: Aging, antioxidant defense, energy status, squalene, n-3 PUFA

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Introduction

Aging is a multifaceted physiological process that leads to gradual loss of ability of an individual to maintain homeostasis. The free radical theory of aging proposes that aging occurs as a consequence of the deleterious effect of free radicals or reactive oxygen species (ROS) produced during the course of cellular metabolism. Mitochondria are an important intracellular source and target of reactive oxygen species. A better understanding of the processes involved in aging has stimulated the search for bioactive substances, which could limit the progression of aging.

In recent times, there is a lot of interest in bioactive molecules of marine origin with potential benefits. It is evident from epidemiological studies (Holub & Holub, 2004; Mori & Beilin, 2004) that there is an inverse relation between fish intake and incidence of age-associated diseases and disorders. It is reported that the presence of eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) in rich quantities in fish are mainly ascribable for this beneficial effect. But, it is very much important to realize the fact that PUFAs are well known for their peroxidative nature, which is highly deleterious to the stabilization of cellular and subcellular membranes. Antioxidant nutrients play a significant role in the body's defense against excess levels of free radicals and delay the onset of aging and age associated degenerative diseases. In particular, antioxidants that can be stored in cellular membranes may be potential candidates for prevention or treatment of disorders involving oxidative damage during aging.

Squalene, a remarkable bioactive substance present in deep-sea shark liver oil in high quantities, belongs to a class of antioxidants called isoprenoids, which neutralize the harmful effects of excessive free radicals produced in the body (Storm et al., 1993). Scientific research and clinical trials have shown that squalene is safe as a dietary supplement in food and capsules and no untoward incidents have been

reported following the use of squalene (Ko et al., 2002). Though these beneficial properties of squalene and n-3 PUFA are promising and well studied, the synergistic effects of squalene and n-3 PUFA on mitochondrial function during aging have not yet been explored. In the present study, the synergistic effects of dietary squalene and n-3 PUFA on mitochondrial function was investigated in liver of rats by virtue of its antioxidant and membrane stabilizing properties by assaying hepatic mitochondrial TCA cycle enzymes and respiratory marker enzymes.

Materials and Methods

Squalene of specific gravity 0.853, refractive index 1.493, saponification value 30, iodine value 344, boiling point 240-245°C was used for the study. PUFA concentrate (Table 1) extracted from sardine oil were used for the experiment. All the other chemicals of analytical grade were procured locally. 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)] used for the study. The control and experimental 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 food [M/s Sai Foods, Bangalore, India] and water *ad libitum*. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee of the Central Institute of Fisheries Technology, Cochin.

Table 1. Fatty acid composition of n-3 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

The animals were divided into two major groups: Group I consisted of 24 normal young rats and Group II consisted of 24 normal aged rats. Seven days after acclimatization, 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 killed and the liver tissue was excised immediately and immersed in ice-cold physiological saline and blotted with filter paper. The hepatic mitochondria were isolated by the method of Johnson & Lardy (1967) and used for the determination of TCA cycle enzymes and respiratory marker enzymes.

The determination of isocitrate dehydrogenase (ICDH, EC 1.1.1.42) was estimated by the method of Bell & Baron (1960). The assay of α-ketoglutarate dehydrogenase (α-KDH, EC 1.2.4.2) assayed by the method of Reed & Mukerjee (1969). Biochemical assay of succinate dehydrogenase (SDH, EC 1.3.99.1) was done by the method of Slater & Borner (1952). Malate dehydrogenase (MDH, EC 1.1.1.37) was estimated by the method of Mehler et al. (1948). Cytochrome-c-oxidase (EC 1.9.3.1) was analysed by the method of Pearl et al. (1963) and NADH dehydrogenase (EC 1.6.99.3) determined by the method of Minakami et al. (1962). The level of ATP content in the liver tissue was determined by the method of Ryder (1985) using Shimadzu LC 10 ATvp HPLC. The results were presented as mean ± standard deviation of determination 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 were analyzed with the help of statistical package program SPSS 10.0 for windows.

Results and Discussion

In the present study, a significant ($p<0.05$) decline was noted in the activities of TCA cycle enzymes (isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase) and respiratory marker enzymes (NADH dehydrogenase and cytochrome-C-oxidase) in the liver mitochondria of Group IIa aged control rats as compared to Group Ia young control rats (Table 2

and Table 3). Also a concomitant reduction in the level of ATP content was registered in the hepatic tissue of aged animals (Table 4). The present observation concurs with an earlier reported investigation (Buddhan et al., 2007), which indicated that the oxidative phosphorylation was operating at a very lower rate in the liver of aged rats despite higher energy demand. ATP has also been reported to reduce oxygen demand (Kang, 2003), which could result in preservation of high-energy phosphate stores during hepatic dysfunction. Incessant oxygen supply is a usual prerequisite for the generation of ATP in the mitochondria for the normal function of the liver. Since diminished oxygen supply is a

Table 2. Synergistic effect of dietary squalene and n-3 PUFA supplementation on the activities of TCA cycle enzymes [Isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase] in the liver mitochondria of young and aged rats

	Young				Aged			
Parameters	Ia (Control)	Ib (PUFA)	Ic (Squalene)	Id (Squalene + PUFA)	IIa (Control)	IIb (PUFA)	IIc (Squalene)	IId (Squalene + PUFA)
ICDH	830 ± 28.62^{a,c}	901 ± 25.74^{b}	845 ± 19.22^{c}	825 ± 18.33^{c}	665 ± 7.38^{d}	590 ± 6.21^{e}	720 ± 34.28^{f}	798 ± 12.27^{a}
α-KDH	76.5 ± 3.82^{a}	95 ± 0.96^{b}	82.75 ± 1.22^{c}	74 ± 2.11^{a,d}	48.4 ± 2.42^{e}	27.12 ± 1.23^{f}	55.16 ± 1.22^{g}	72 ± 0.84^{d}
SDH	39.2 ± 1.96^{a}	72 ± 0.96^{b}	42.6 ± 0.58^{c}	36 ± 1.8^{d}	20.95 ± 1.04^{e}	9.42 ± 0.10^{f}	24.72 ± 0.85^{g}	32 ± 1.6^{h}
MDH	385 ± 19.25^{a}	495 ± 19.8^{b}	375 ± 4.63^{a}	379 ± 18.95^{a}	263 ± 13.15^{c}	170 ± 8.5^{d}	283 ± 8.10^{c}	370 ± 5.69^{a}

Results are mean ± SD for six animals. Values that have a different superscript letter (a,b,c,d,e,f,g,h) differ significantly ($p<0.05$) with each other. Values expressed: Isocitrate dehydrogenase, nmol of α-ketoglutarate formed min^{-1} mg^{-1} protein; α-ketoglutarate dehydrogenase, nmol of ferro cyandide formed min^{-1} mg^{-1} protein; succinate dehydrogenase, μmol of succinate oxidized min^{-1} mg^{-1} protein; malate dehydrogenase μmol of NADH oxidized min^{-1} mg^{-1} protein

Table 3. Synergistic effect of dietary squalene and n-3 PUFA supplementation on the activities of respiratory marker enzymes in the liver mitochondria of young and aged rats

	Young				Aged			
Parameters	Ia (Control)	Ib (PUFA)	Ic (Squalene)	Id (Squalene + PUFA)	IIa (Control)	IIb (PUFA)	IIc (Squalene)	IId (Squalene + PUFA)
NADH Dehydrogenase	39.4 ± 1.97 ^{a}	54 ± 2.57^{b}	42.7 ± 0.49^{c}	35.15 ± 1.67^{d}	25.3 ± 1.26^{e}	14 ± 0.7^{f}	29.6 ± 1.45^{g}	34 ± 0.52^{d}
Cytochrome C- Oxidase	4.19x10^{2}±0.2^{a}	7.51x10^{-2} ±0.11^{b}	4.81x10^{2}±0.2^{c}	4.30x10^{-2} ±0.19^{a}	3.11x10-^{2}±0.15^{d}	1.42x10^{2}±0.0^{e}	3.35x10^{-2} ±0.15^{d}	4.12x10^{-2}±0.05^{a}

Results are mean ± SD for six animals. Values that have a different superscript letter (a,b,c,d,e,f,g) differ significantly ($p<0.05$) with each other. Values expressed: NADH-dehydrogenase, μmol of NADH oxidized min^{-1} mg^{-1} protein; cytochrome-C-oxidase, O.D. min^{-1} mg^{-1} protein

Table 4. Effect of dietary squalene and n-3 PUFA supplementation on ATP content in the liver tissue 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)
ATP	5 ± 0.25^{a,c}	5.54 ± 0.32^{b}	5.43 ± 0.30^{b,c}	5.82 ± 0.34^{b}	3.45 ± 0.28^{d}	2.79 ± 0.14^{e}	3.94 ± 0.19^{f}	4.89 ± 0.25^{a}

Results are mean ± SD for six animals. Values that have a different superscript letter (a,b,c,d,e,f) differ significantly ($p<0.05$) with each other. Values expressed: ATP, n mol g^{-1} wet tissue

common occurrence during process, it might have ultimately resulted in a rapid reduction in the level of mitochondrial respiration in the hepatic tissue. Dietary *per se* intake of n-3 PUFA has aggravated the age-associated aberrations in the mitochondrial function in aged rats. However, the combined dietary supplementation of squalene and polyunsaturated fatty acid concentrate significantly ($p<0.05$) attenuated the above-mentioned age-related aberrations and maintained the liver mitochondrial energy status at near normal, indicating the ability of squalene to inhibit the n-3 PUFA mediated uncoupling between respiration and oxidative phosphorylation during aging.

In the present investigation, the dietary squalene intake probably rendered the protective action on mitochondrial function in the oxygen deprived liver through motivating mechanism. Squalene is not only an effective antioxidant, but also serves as a good oxygen provider into the cell system. During this process, it liberates oxygen for rejuvenation of cells (Hayashi et al., 2003). Experimental studies by Yokota (1995) on the chemical behaviour of this isoprenoid have shown that squalene is naturally lacking 12 hydrogen atoms in its original form for it to be stable (the stable compound is $C_{30}H_{62}$) and will "capture hydrogen atoms" from any source available to make it stable and saturated. $C_{30}H_{50}$ (squalene) may react with water (H_20) for the release of molecular oxygen.

Hence, it is possible that squalene is capable of supplying oxygen necessary for the retrieval of the aged liver.The results of the present investigation indicated that the combined dietary intake of squalene and PUFA concentrate is more effective in ameliorating the age associated aberrations compared to *per se* supplementation. It is concluded that dietary squalene and n-3 polyunsaturated fatty acids (PUFA) may be an effective therapeutic method in treatment of age associated disorders, where free radicals are a major causative factors.

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