THE EFFECT OF FISH OIL ON OXIDANT/ANTIOXIDANT STATUS IN DIABETIC RATS THROUGH THE REDUCTION OF ARACHIDONIC ACID IN THE CELL MEMBRANE

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ABSTRACT

Background: Dietary omega-3 fatty acids directly affect arachidonic acid (AA) metabolism because they displace AA from membranes and compete with it for the enzymes that catalyze the biosynthesis of thromboxanes, prostaglandins and leukotrienes.

Objective: This study aimed to evaluate the role of fish oil supplementation in reducing the free radicals production through the reduction of arachidonic acid level in erythrocyte membrane in experimental diabetic rats.

Methods: Forty eight male albino rats were used in this study and divided into four groups :control, fish oil, diabetic and treated groups. Fish oil and treated groups were administered fish oil in a dose of 1.2 ml /kg bw/day orally for 8 weeks. Urinary 8-hydroxyguanosine and erythrocyte membrane arachidonic acid were estimated by HPLC. Also, urinary isoprostanes and erythrocyte membrane superoxide dismutase were determined.

Results: Our results indicated that hyperglycemia in diabetic rats significantly increased urinary 8-hydroxyguanosine and isoprostanes and erythrocyte membrane arachidonic acid, whereas, supplementation of fish oil significantly decreased these values in treated group.

Conclusion: Fish oil supplementation has an important role in attenuating the elevation of arachidonic acid (omega-6) in cell membrane phospholipids resulting in a reduction in free radicals production.

Keywords: Fish oil, Diabetes Mellitus, 8-hydroxyguanosine, Isoprostanes, Arachidonic acid.

INTRODUCTION

During diabetes, persistent hyperglycemia causes increased production of free radicals especially reactive oxygen species (ROS), for all tissues from glucose auto-oxidation and protein glycosylation [1]. Abnormally high levels of free radicals and simultaneous decline of antioxidant defense systems can lead to the damage of cellular organelles and enzymes, increased lipid peroxidation and development of complications of diabetes mellitus [2]. Isoprostanes are a proposed biomarker of oxidative stress, they are produced by nonenzymatic peroxidation of arachidonic acid (AA; 20:4x6), esterified to phospholipids and subsequently released, possibly by a phospholipase. They circulate in the blood stream and are finally excreted in urine [3]. Also oxidative modified DNA in the form of 8-Hydroxy-2-Deoxyguanosine (8-OHdG) can be quantified to indicate the extent of DNA damage [4]. Urinary 8-OHdG an oxidative damage nucleoside, has been widely used as a marker for evaluating _in vivo_ oxidative stress [5]. Food derived antioxidants have a strong potential for long term use as chemo-preventive agents in disease states involving oxidative stress [6]. It was found that, dietary omega-3 fatty acids directly affect AA metabolism because they displace AA from membranes and compete with it for the enzymes that catalyze the biosynthesis of thromboxanes, prostaglandins and leukotrienes [7]. So, consuming food enriched in omega-3 fatty acids (such as fish oil) is diminished potential for cells like monocytes, neutrophils and esinophils to synthesize these powerful arachidonic acid–derived mediators of inflammation and a diminished potential for platelets to produce the prothrombotic agent thromboxane A2 [8]. From this light, we aimed to evaluate the role of fish oil supplementation in reducing the free radicals production through the reduction of arachidonic acid level in erythrocyte membrane in experimental diabetic rats.

MATERIALS AND METHODS

Materials, Chemicals

Streptozotosin (STZ) and 8-hydroxyguanosine & arachidonic acid standards (high performance liquid chromatography (HPLC) grade) were purchased from Sigma Chemicals Co. (Munih, Germany). All other chemicals were HPLC grade and purchased from ALDRICH, Germany.

Experimental animals

Male albino rats weighting 180 to 200 g were obtained from the animal house of National Research Center, Giza, Egypt.

Methods

The guidelines of the ethical care and treatment of the animals followed the regulations of the ethical committee of the National Research Center (NRC).

Induction of diabetes mellitus

STZ was dissolved in 50 mM sodium citrate (pH 4.5) solution containing 150 mM NaCl. The solution (6.0 mg/0.5 ml/100 g body weight (bw)) was subcutaneously administrated in rats, fasting blood sugar was estimated after 3 days to confirm the development of diabetes mellitus [9].

Experimental design

Forty eight male albino rats were housed in individual suspended stainless steel cages in a controlled environment (22 to 25°C) and 12 h light/12 h dark with food and water freely available, and were divided randomly into four groups ; 12 rats in each group as follow:

1. Group I (control group): healthy rats received 1.2 ml corn oil/kg bw/day orally.

2. Group II (fish oil group): healthy rats received 1.2 ml fish oil/kg bw/day orally.

3. Group III (diabetic group): diabetic rats received 1.2 ml corn oil/kg bw/day orally.

4. Group IV (treated group): diabetic rats received 1.2 ml fish oil/kg bw/day orally [10].

After the experimental period (8 weeks), 24 h urine was collected from each animal for estimation of urinary 8-hydroxyguanosine, F2 isoprostane and creatinine.

Fasting blood samples were withdrawn from the retro-orbital venous plexus under light anaesthesia by diethyl ether [11], and collected in tubes containing sodium fluoride for blood glucose and heparinized tubes for erythrocyte membrane arachidonic acid and superoxide dismutase (SOD) estimation.

Ghost preparation

The method used for erythrocyte ghost preparation was based on washing of backed RBCs by isotonic phosphate buffer and hemolysis of RBCs for removal of hemoglobin by hypotonic phosphate buffer (pH was adjusted at 7.4) [12,13].

Erythrocyte membrane lipids extraction

Lipids in erythrocyte membrane were extracted with chloroform: methanol method [14] modified from the method described by Bligh and Dyer [15].

Biochemical analysis

Fasting blood sugar was determined using enzymatic colorimetric method, Centronic, Germany, according to Trinder [16].

Membrane superoxide dismutase (SOD) as antioxidant enzyme was evaluated according to the method described previously [17].

Urinary F2 isoprostane was estimated by enzyme immunoassay (ELISA) by a kit derived from Cayman Ann Arbor, USA [18], also urinary creatinine was estimated by kinetic method [19].

Estimation of urinary 8-hydroxyguanosine by HPLC

8-hydroxyguanosine (8-OHdG) was estimated by HPLC system, Agilent Technologies 1100 series, equipped with a quaternary pump (Quat Pump, G131A model) after modification of the method described by Kim et al. [20].

Standard preparation

Standard was dissolved in deionized water, 20 µl from serial dilution of standard were injected in HPLC to draw a standard curve with different concentration.

Sample extraction

8-OHdG was extracted from 1 ml urine. The eluents were dried under ultrapure N2 stream and were reconstituted in 5 ml deionized water. 20 µl from each sample were injected in HPLC.

The concentration of urinary 8-OHdG was calculated from the standard curve and divided by the urinary creatinine.

HPLC condition

HPLC column C18 (250 × 4.6, particle siz e 5 µl) using mobile phase acetonitrile/methanol/phosphate buffer (25/10/965) v/v. Phosphate buffer was prepared by dissolving 8.8 g of potassium dihydrogen phosphate in 1000 ml deionized water and pH was adjusted at 3.5 using phosphoric acid. The buffer was then filtered 2 times before being used at a flow rate of 1 ml/min using electrochemical detector with cell potential of 600 mV [21].

Arachidonic acid estimation by HPLC

Sample preparation

Cell membrane was homogenized in 2 % acetic acid : ethyl ether mixture (2:1 volume ratio). The solution was then filtered and centrifuged at 500 xg, the organic phase was evaporated to dryness. The extract was dissolved in 200 µl acetonitrile [22].

HPLC condition

This method was carried out according to the method described previously [23]. HPLC column C 18 (260 X 4.6, particle size 5 µl), mobile phase was acetonitrile / water mixture (70/30) v/v by isocratic elution with flow rate 1 ml / min and 200 nm wave length. Serial dilutions of standards were injected and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentration in samples was obtained from the curve.

Statistical analysis

Results were expressed as mean ± standard error. Data were analyzed by independent sample t test (SPSS) version 15 followed by (LSD) test to compare significance between groups. Difference was considered significant when P value <0.05.

RESULTS

In the current study, there was a significant increase in blood glucose level in diabetic group compared to control while this value was significantly decreased by fish oil supplementation in treated group compared to diabetic although it still significantly increased compared to control group (Fig.1).
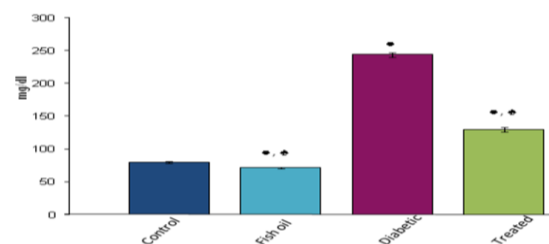

Fig. 1: Fasting blood glucose levels in different studied groups

●:Significant difference compared to control group.

♠:Significant difference compared to diabetic group.

Erythrocyte membrane superoxide dismutase (SOD) was significantly decreased whereas a concomitant significant increase in urinary isoprostanes and 8-OHdG was observed in diabetic rats compared to control (Fig 2-4). Fish oil supplementation, significantly increased SOD in treated group compared to diabetic although it still significantly decreased compared to control group (Fig.2).
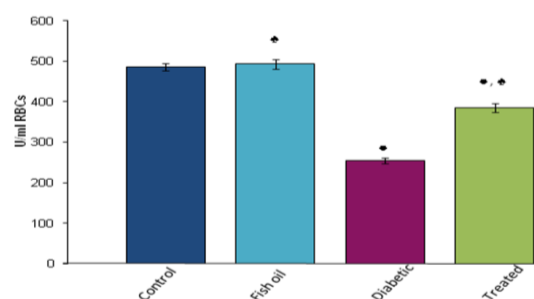

Fig.2: Superoxide dismutase levels in different studied groups

●:Significant difference compared to control group.

♠:Significant difference compared to diabetic group.

Administration of fish oil in this study, significantly decreased both urinary isoprostanes and 8-OHdG in treated group compared to diabetic, although 8-OHdG was still significantly increased compared to control group (Fig 3-4). In diabetic group, AA was significantly increased compared to control but it was decreased again in treated group to become more or less near the control group (Fig 5).

DISCUSSION

STZ-induced diabetes provides a relevant example of endogenous chronic oxidative stress and hyperglycemia. Thus, we evaluated the oxidative stress induced by STZ in rats and examined the potential role of fish oil against the changes STZ induced. Increased oxidative stress could be one of the common pathogenic factors of diabetic complications [24], which is generally attributed to the formation of highly reactive OH· that leads to severe oxidative damage of the cell's components like lipids, proteins and DNA [25].
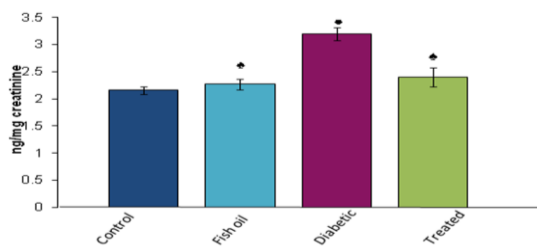
Fig.3: Urinary isoprostanes levels in different studied groups

●:Significant difference compared to control group.

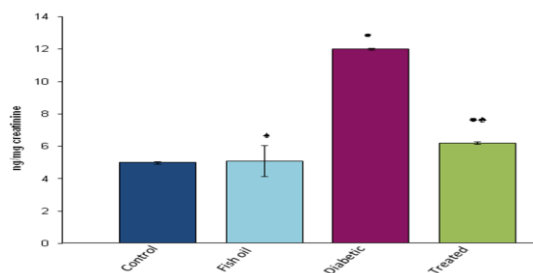♠:Significant difference compared to diabetic group.

Fig. 4: Urinary 8-hydroxydeoxyguanosine levels in different studied groups

●:Significant difference compared to control group.

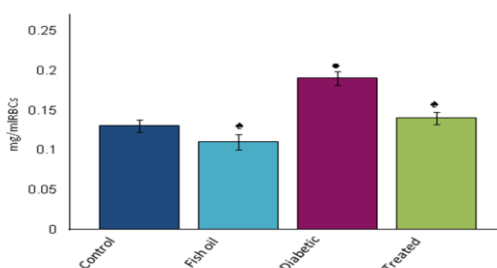♠:Significant difference compared to diabetic group.

Fig. 5: Arachidonic acid levels in different studied groups

●:Significant difference compared to control group.

♠:Significant difference compared to diabetic group.

The major targets of these damaging species are the long chain polyunsaturated fatty acids (LC-PUFAs) of cellular phospholipids, which are particularly prone to attack because of the arrangement of double and single bonds. The resultant lipid peroxide frequently decomposes to radicals [26], which react with most biological molecules, including proteins and lipids [27]. The reduction of erythrocyte membrane superoxide dismutase (SOD) in diabetic group in this study was in agreement with the previous study [28], which indicated that, the activity of SOD decreased significantly in diabetic rats. They suggested that, the reduction in SOD activity could be due to increased utilization for scavenging free radicals. A decrease in SOD can lead to an excess availability of superoxide anion and hydrogen peroxide in biological system, which in turn generates OH⁻, resulting in initiation and propagation of lipid peroxidation [29], elevating urinary isoprostanes as was found in this study. In agreement, Ulven *et al.* [30], demonstrated that, an acute hyperglycaemic leads to an increase of F2-isoprostanes generation in type 2 diabetes mellitus. This elevation is due to the generation of free radicals from the arachidonic acid (omega-6) in cell membrane phospholipids [31]. Araki and Nishikawa [24] found a positive correlation between HbA1C and urinary excretion of 8-OHdG which reflected mitochondrial oxidative damage. Other study [32] indicated that, the urinary content of 8-OHdG represents an average rate of oxidative damage to guanine in the form of the free nucleotide (dGTP) and in DNA. Moreover, streptozotocin prevents

DNA synthesis in mammalian cell by inhibiting the substrate connection to the DNA or inhibiting many of the enzymes involved in DNA synthesis [33]. Two clinical investigations reported a significant increase in plasma and tissue AA concentrations in patients compared with controls [34,35], which could be the result of enhanced lipid peroxidation in disease [36].Alternatively, the increased AA levels could be due to elevated desaturase activity on linoleic acid (LA) leading to increased formation of prostaglandins and other lipoxygenase products.

Administration of fish oil in this study, significantly decreased both urinary isoprostanes and 8-OHdG whereas it significantly increased antioxidant enzyme (SOD) in treated group compared to diabetic. In agreement, Makni *et al.* [37] indicated that, feeding seeds rich in PUFAs, significantly increased the activities of antioxidant enzymes such as superoxide dismutase (SOD) level in plasma and liver of hypercholesterolemic rats. It was found that, fish oil contains a free radicals scavenging activity, which could exert a beneficial effect against pathological alterations caused by the presence of O^{2-} and OH^-. The increased activity of SOD accelerates dismutation of O^{2-} to hydrogen peroxide [28].

This action could involve mechanisms related to scavenging activity of fish oil, thus, fish oil contains high amount of unsaturated fatty acids, especially omega-3 [38], which up regulate gene expression of antioxidants enzymes and down regulate gene associated with production of reactive oxygen species [39]. In this study oral supplementation with fish oil which is a good source of omega-3 fatty acids significantly decreased AA level in erythrocyte membrane. When humans ingest fish or fish oil as was found in our experiment, the ecosapentanoic acid (EPA) and decosahexanoic acid (DHA) from the diet partially replace the omega-6 fatty acids, especially AA, in the membranes of probably all cells, but especially in the membranes of platelets, erythrocytes, neutrophils, monocytes, and liver cells leading to the reduction of oxidative stress parameters such as isoprostanes and 8-OHdG.

Whereas cellular proteins are genetically determined, the polyunsaturated fatty acid (PUFA) composition of cell membranes is to a great extent dependent on the dietary intake [40]. From this light, we concluded that, fish oil supplementation has an important role in attenuating oxidative stress in diabetic rats. This finding may be due to the fact that fish oil contains high amount of omega-3 fatty acids in the form of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are very beneficial fatty acids that have antioxidant and anti-inflammatory effects in addition to their incorporation in the cell membrane, when these fatty acids incorporate into cell membrane the percent of other fatty acids such as arachidonic acid (omega-6) in cell membrane phospholipids decreased resulting in a reduction in free radicals production.

ACKNOWLEDGEMENT

REFERENCES

1. Robertson RP. Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. J. Biol. Chem.,2004; 279: 42351–42354.
2. Maritim AC, Sanders RA, Watkins JB. Diabetes, oxidative stress and antioxidants. J. Biochem. Molec. Toxicol., 2003; 17: 24–38.
3. Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ. Non cyclo-oxygenase derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. Proc. Natl. Acad. Sci. USA, 1992; 89: 10721– 10725.
4. Cooke MS, Lunec J, Evans MD. Progress in the analysis of urinary oxidative DNA damage. Free Rad. Biol. Med., 2002; 33: 1601– 1614.
5. Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2-deoxyguanosine (8-OHdG): A Critical Biomarkerof Oxidative Stress and Carcinogenesis Journal of Environmental Science and Health, Part C,2009; 27: 120-139.
6. Mckim SE, Kono A, Gabele E, Uesugi T, Froh M, Sies H, Thurman RG,Arteel GE. Cocoa extract protects against early alcohol

induced liver injury in the rat. Arch. Biochem. Bioassay, 2002; 406: 40-46.

7. Lindsberg P J, Hallenbeck J M, Feuerstein G. Platelet-activating factor in stroke and brain injury. Ann Neurol., 1991; 30(2): 117-29.

8. Calder P C. n-3 polyunsaturated fatty acids, inflammation and inflammatory diseases. Am J Clin Nutr.2006; 83:1505S-19S.

9. Uchiyama S, Yamaguchi M. Alteration in serum and bone component findings induced in streptozotocin-diabetic rats is restored by zinc acexamate. International. Journal of Molecular medicine, 2003; 12:949-954.

10. Taylor GC, Noto DA, Stringer DM, Froese S, Malcolmson L. Dietary milled flaxseed and flaxseed oil improve n-3 fatty acid status and do not affect glycemic control in individuals with well-controlled type 2diabetes. Journal of the American College of Nutrition, 2010; 29 (1):72-80.

11. Madway W, Prier LE, Wilkinson JS. A Textbook of Veterinary Clinical Pathology. The Willims and wilking co.Battimore,1969.

12. Post RL, Merritt CR, Albright CD. Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. J. Biol. Hematol.,1960; 235:1796-1802.

13. Dodge JT, Mitchell C, Hanahan DJ. The preparation and chemical characteristics of haemoglobin – free ghosts of human erythrocytes. Acta. Bioquim. Clin., 1962; 2:404-425.

14. DeGier J, Van Deenen LL. Phospholipid and fatty acid characteristics of erythrocytes in some cases of anemia. Br J Haematol,1964; 10:246.

15. Blight EG, Dyer WJ. A rapid method of total lipid extraction and purification. Candian. Journal of Biochem Physiol, 1959; 37: 911-915.

16. Trinder P. Determination of glucose in blood using glucose oxidase with alternative oxygen acceptor. Annals Clinical Biochemistry,1969; 6: 24-27.

17. Nishikimi M, Roa NA, Yogi K. Determination of superoxide dismutase. Biochem. Bioph. Res. Common.,1972; 46: 849-854.

18. Montuschi P, Barnes PJ, Roberts LJ. Isoprostanes: Markers and mediators of oxidative stress. FASEB J.,2004; 18: 1791-1800.

19. Larsen K. Creatinine assay by a reaction-kinetic principle. Clin. Chim. Acta.,1972; 41:209-217.

20. Kim M, Moon H, Hong S. Determination of urinary 8-hydroxy-2-deoxy guanosine as a DNA damage marker. Am. Clin. Lab.,2001; 42-45.

21. El-Khayat Z, Rasheed W, Ramzy T, Hussein J, Agaiby M, Morsy S, Morsy F, Shaffie N. Protective effect of garlic oil against liver injury in experimental animals. J. Med. Plants Res.,2010; 4(22): 2359-2369.

22. Lim CK. HPLC of small molecules. Tracey B, Lipids, IRL Press, Oxford, 1986.

23. El-Khayat Z, Abo El-Matty D, Rasheed W, Hussein J, Shaker O, Raafat J. Role of cell membrane fatty acids in insulin sensitivity in diabetic rats treated with flaxseed oil. Int J Pharm Pharm Sci, 2013; Vol 5, Suppl 2, 146-151.

24. Araki E, Nishikawa T. Oxidative stress: A cause and therapeutic target of diabetic complications. J. Diabetes Invest., 2010;1: 90-96.

25. Mckillop IH, Schrum, LW. Alcohol and liver cancer. Alcohol.,2005; 35(3): 195-203.

26. Spiteller G. Linoleic acid peroxidation the dominant lipid peroxidation process in low density lipoprotein and its relationship to chronic diseases. Chem. Phys. Lipids.,1998; 95: 105–162.

27. Ryu JK, Lee T, Kim D, Park I., Yoon S, Lee H, Song S, Suh J. Free radical scavenging activity of korean red ginseng for erectile dysfunction in non-insulin-dependent diabetes mellitus rats. Urology, 2005; 611-615.

28. Murugan P, Pari L. Influence of tetrahydrocurcumin on erythrocyte membrane and antioxidant status in experimental type 2 diabetic rats. J. Ethnopharmacol., 2007; 113: 479-486.

29. Kumuhekar HM, Katyane SS. Altered kinetic attributes of Na+/K+ ATPase acivity in kidney, brain and erythrocyte membrane in alloxan diabetic rats. Indian J. Exp. Biol.,1992; 30: 26–32.

30. Ulven S, Kirkhus B, Lamglait A, Basu S, Elind E, Haider T, Berge K, Vik H, Pedersen JI. Metabolic effects of krill oil are essentially similar to those of fish oil but at lower dose of EPA and DHA in healthy volunteers. Lipids., 2011; 46(1): 37-46.

31. Subash P, Gurumurthy P, Sarasabharathi A, Cherian KM. Urinary 8-OHdG: A marker of oxidative stress to DNA and total antioxidant status in essential hypertension with South Indian population. Indian J. Clin. Biochem.,2010; 25(2): 127-132.

32. Svoboda P, Ko S, Cho P, Yoo S, Choi S, Ye S. Neopterin, a marker of immune response, and 8-hydroxy-2-deoxyguanosine, a marker of oxidative stress, correlate at high age as determined by automated simultaneous high performance liquid chromatography analysis of human urine. Analy. Biochem.,2005; 383: 236-242.

33. Akbarzadeh A, Norouzian D, Mehrabi MR, Jamshidi SH, Farhangi A, Verdi AA, Mofidian1 SMA, Rad B L.. Induction of diabetes by streptozotocin in rats. Indian J. Clin. Biochem., 2007;22 (2): 60-64.

34. Neoptolemos JP, Husband D, Imray C, Rowley S, Lawson N. Arachidonic acid and docosahexaenoic acid are increased in human colorectal cancer. Gut., 1991; 32:278-281.

35. Hendrickse CW, Kelly RW, Radley S, Donovan IA, Keighley MR. Neoptolemos JP. Lipid peroxidation and prostaglandins in colorectal cancer. Br J Surg., 1994; 81:1219-1223.

36. Skrzydlewska E, Sulkowski S, Koda M, Zalewski B, Kanczuga- Koda L, Sulkowska M. Lipid Peroxidation and antioxidant status in colorectal cancer. World J Gastroenterol,2005; 11:403-406.

37. Makni M, Fetoui H, Gargouri NK, Garoui EM, Jaber H, Makni J, Boudawara V T, Zeghal N. Hypolipidemic and hepatoprotective effects of flax and pumpkin seed mixture rich in n-3 and n-6 fatty acids in hypercholesterolemic rats. Food Chem. Toxicol.,2008; 46: 3714–3720.

38. Maddock TD, Bauer ML, Koch K. Effect of processing flax in beef feed lot diets on performance, carcass characteristics, and trained sensory panel ratings. J. Anim. Sci., 2006; 84: 1544-1551.

39. Harding AH, Agil A, Mykkanen L. Habitual fish consumption and glycated haemoglobin. The EPIC-Norfolk study. Eur. J. Clin. Nutr.,2004; 58(2): 277–284.

40. Simopoulos A P. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Experimental Biology and Medicine., 2008; 233:674.