# Effect of *in vitro* Stability of Dietary Fish Oil on Lipid Peroxidation and Prostanoids *in vivo*

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## ABSTRACT

Dietary supplementation of rats with fish oil for 18 days resulted in signs of lipid peroxidation, with increased malondialdehyde production in plasma and myocardium. This increase in malondialdehyde could be completely prevented by supplementing the fish oil with a new natural antioxidant mixture (Pufanox®) which is known to markedly increase the in vitro stability of fish oils. Addition of Pufanox® to fish oil also tended to increase the ratio between the vasodilator prostacyclin and the vasoconstrictor thromboxane $A_2$. The concentration of vitamin E decreased, both in plasma and in the heart after the period of fish oil ingestion, indicating that the fish oil used contained too little vitamin E. Plasma cholesterol and triglyceride levels decreased markedly after fish oil supplementation but with no apparent difference between fish oil with or without Pufanox®, probably due to the insufficient content of vitamin E. The results obtained emphasize the importance both of the in vitro stability of the fish oil given and of the amount of vitamin E added.

## INTRODUCTION

There is epidemiological, clinical and experimental support for the belief that fish oils containing omega-3 fatty acids have protective qualities against both cardiovascular disease and inflammatory complaints (4).

Omega-3 fatty acids in fish oils are highly unsaturated and potentially unstable compounds. Consequently, exposure of most fish oils to air quickly leads to their deterioration, with consequent increase in rancidity indexes e.g. for peroxide and anisidine. Addition of a new mixture of natural antioxidants, Pufanox®, to fish oil has been shown to increase their stability considerably. Thus, Pufanox® increased the lag to rancidity after exposing the oil to air at room temperature, from a few days up to 200 days (8).

In the present investigation we examined the effect of a natural fish oil, both with and without Pufanox®, on lipid peroxidation and prostanoids.

# MATERIAL AND METHODS

**Animals.** Male pathogen-free Sprague-Dawley rats, weighing 204-233 g (B&K Universal AB, Sollentuna, Sweden) were caged in pairs in a room with controlled temperature (20-21°), lighting (lights on between 0600 and 1800 h) and humidity (50-70%). All procedures and care of the animals had been approved by the local Animal Care and Use Committee. In experiment I the rats were allowed free access to tap water and were given pellets (enriched with 7% butter, 7% fish oil with or without Pufanox®) totalling 25 g per day and rat. In experiment II the animals were allowed free access to standard pellets and to normal tap water or tap water with vitamin E added. After 18 days (experiment I) or 14 days (experiment II) the rats were weighed, anesthetized with Pentobarbital sodium (60 mg/kg body weight) given intraperitoneally, and then exsanguinated via the abdominal aorta. Serum was prepared from whole blood kept in a +37°C water bath for 1 h, centrifuged for 20 min at 500 $g$ at +4°C and then stored at -70°C. Plasma was prepared immediately by centrifugation of blood for 15 min at 500 $g$ at +4°C, in tubes containing EDTA and then stored at -70°C. The heart was excised, divided into small portions and stored at -70°C.

**Diets.** Standard pellets used for enrichment contained 57% carbohydrates, 17% protein, 1.4% fat and 74 mg vitamin E/kg. The control group in experiment I was given pellets enriched with 7% butter. One group of rats received pellets enriched with 7% fish oil containing vitamin E (1.1 IU/g), while the other group was given pellets with 7% fish oil containing a recently developed mixture of natural antioxidants (Pufanox®, Cardinova, Uppsala, Sweden, patent pending) and vitamin E (1.7 IU/g). Each rat received about 1.75 g fish oil daily. In experiment II the control group had free access to tap water, while the two experimental groups were given tap water containing vitamin E, corresponding to 10 IU and 30 IU daily.

**Malondialdehyde in plasma.** Lipid peroxidation in plasma was measured by determining the concentration of malondialdehyde (MDA) by HPLC, as described previously (9). EDTA-blood (1 mL) was immediately mixed with 10 µl butylated hydroxytoluene (BHT, 227 mmol/L, in ethanol) and the plasma was stored at -70°C until analysed. 0.5 mL of the sample was boiled with 0.75 mL phosphoric acid (0.15 mol/L) and 0.25 mL thiobarbituric adid (TBA) (42 mmol/L) in a water bath (100°C) for 1 h and then placed on ice. 0.5 mL of the boiled sample was neutralized by adding of 0.5 mL a methanol-NaOH solution and then centrifuged at 9000 $g$ for 5 min at +4°C. Results are expressed as mmol/L.

**Malondialdehyde in myocardium.** MDA was determined as the TBA - MDA complex (9). Briefly, a piece of heart muscle (0.1-0.3 g) was homogenized (1:10) in Tris/KCl buffer (0.2 mol/L/0.16 mol/L, pH 7.4) and incubated in a water bath (+37°C) for 1 h. 0.5 g of the incubated heart homogenate was boiled with 0.75 mL phosphoric acid (0.15 mol/L) and 0.25 mL TBA (42 mmol/L) in a water bath (100°C) for 1 h and then placed on ice. After adding of 0.5 mL of a methanol-NaOH solution to 0.5 mL of sample, the samples were centrifuged at 9000 $g$ for 5 min at

214

4°C, and the supernatants were measured in a spectrophotometer at 532 nm, using tetra-ethoxypropane (TEP) as standard. The results are expressed as nmol/g wet tissue. The MDA concentrations in the rat myocardium did not differ significantly, whether analysed by HPLC or spectrophotometrically.

**Vitamin E concentrations in serum and plasma.** 500 μL serum was added to 500 μL ethanol containing 0.005% BHT. The mixture was vortexed for 5 s, 2 mL hexane was added and the mixture was again vortexed, for 3 min. After centrifugation for 10 min at 1300 $g$, the hexane layer was removed and evaporated to dryness under nitrogen and redissolved in 2 mL methanol. The test tubes were centrifuged once again for 10 min at 1300 $g$ and 1 mL of the supernatant was removed to a small vial. Vitamin E levels were analysed with HPLC at 292 nm as described above. Plasma was treated and analysed by the same procedure.

**Vitamin E concentration in myocardium.** The concentration of α-tocopherol in heart muscle was assayed as described by Lang et al. in 1986 (3). Briefly, 100 mg heart muscle was first homogenized in 1 mL distilled water containing 10 μL of ethanolic BHT (227 mmol/L). Thereafter, 1 mL SDS (sodium dodecyl sulphate) (0.1 mol/L) was added and homogenization was completed. The sample was transferred to a test tube and the homogenizer was rinsed with ethanol, which was then combined with the sample. The mixture was vortexed for 30 s, 2 mL hexane was added, and the mixture was again vortexed, for 2 min. After centrifugation for 5 min at 1000 $g$, the hexane layer was removed and evaporated to dryness under nitrogen and redissolved in 2 mL methanol. The test tubes were again centrifuged, this time for 10 min at 1300 $g$ and 1 mL of the supernatant was transferred to a small vial. The tocopherol isomers were separated by RP (Reverse Phase)-HPLC on a 10 cm column packed with Nucleosil C18 (5 μm). The mobile phase was methanol pumped at a flow rate of 1.0 mL/min and the α-tocopherol level was detected and quantified at 292 nm. The results are expressed as nmol/g wet tissue. The coefficients of variation in samples taken from a single batch of myocardial homogenate were about 2-4% ($n$=30 in three separate experiments). The recovery of α-tocopherol added to myocardial homogenate was >90% as compared with direct injection into the RP-HPLC system.

**6-keto-prostaglandin $F_{1\alpha}$ and thromboxane $B_2$ in myocardium.** A piece of heart muscle (about 100 mg) was washed in cold Hanks' balanced salt solution (HBSS, pH 7.4) without calcium and magnesium (four times) before it was incubated in a water bath at +37°C in HBSS (pH 8.2) with calcium and magnesium in presence or absence of indomethacin (0.3 mmol/L). The samples were gently shaken continuously. After 30 min the incubation medium was removed and stored at -70°C and the remaining muscle was weighed. Concentrations of thromboxane $B_2$ and 6-keto-prostaglandin (PG) $F_{1\alpha}$ were determined by radio-immunoassay using ^{3}H-labelled antigen and antirabbit antibody, as described by Saldeen et al. (7). The sensitivity of the assays was about 37 ng/L. The cross-reactivity of 6-keto-PGF$_{1\alpha}$ with PGF$_{2\alpha}$

was 2.6%, with $PGE_1$ 1.9%, with $TxB_2$ 1.4%, with $PGE_2$ 1.1%, with $PGF_{1\alpha}$ 0.8%, and with other compounds, <0.5%. The cross-reactivity of thromboxane $B_2$ with $PGD_2$ was 3.9% and with other compounds, <0.5%. The results are expressed as nmol/g wet tissue.

**6-keto-prostaglandin $F_{1\alpha}$ and thromboxane $B_2$ in serum.** Concentrations of thromboxane $B_2$ and 6-keto-prostaglandin $F_{1\alpha}$ in serum were determined with the same assays as used for the incubation medium above. The results are expressed as ng/L.

**Triglycerides and cholesterol in plasma.** Triglycerides and total cholesterol in plasma were measured by enzymatic colorimetric methods at 500 nm (Peridochrom® Triglycerides GPO-PAP and Monotest® Cholesterol CHOP-PAP, Boehringer Mannheim, Sweden).

## RESULTS

Experiment I

**Malondialdehyde in myocardium.** After 18 days of dietary supplementation with fish oil, myocardial MDA production in vitro was greater in rats fed fish oil without Pufanox® ($p<0.05$) than in those given fish oil with Pufanox®, or in the control group given butter. MDA production in rats fed fish oil with Pufanox® was not higher than in control rats (Fig. 1).

**Malondialdehyde in plasma.** Plasma MDA concentrations were significantly greater in rats given fish oil without Pufanox® ($p<0.001$) than in control rats, whereas MDA levels in rats fed fish oil with Pufanox® did not differ from control values (Fig. 1)
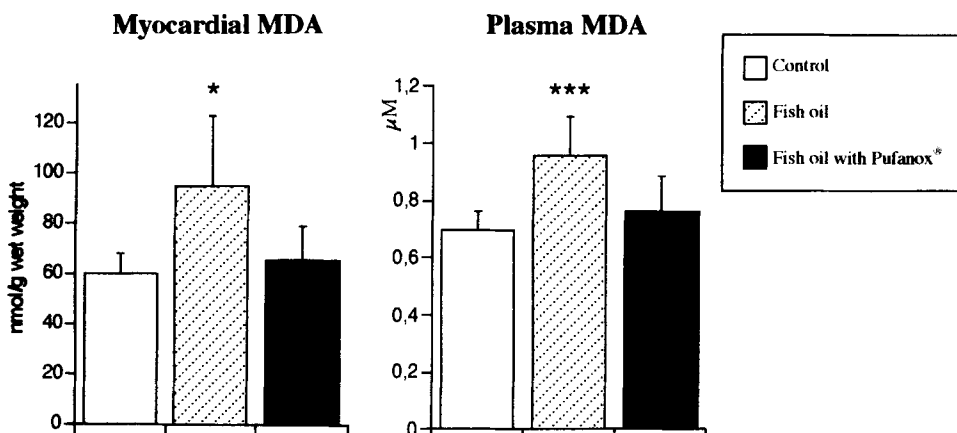


**Fig 1.** Effects of 18 days of dietary supplementation with fish oil with or without Pufanox® on malondialdehyde concentration in the rat heart (nmol/g wet weight) and in plasma (µM). Mean ±SEM, $n=6$ in each group. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with controls.

216

**Thromboxane $B_2$ and 6-keto-prostaglandin $F_{1\alpha}$ in serum.** Significantly lower concentrations of $TxB_2$ and $PGF_{1\alpha}$ were measured in serum of rats given fish oil with ($p<0.01$) and without Pufanox® ($p<0.001$) than in rats in the control group (Fig. 2). There were no significant differences in $TxB_2$ level between these two fish oil dietary groups. Concentrations of $PGI_2$ in serum after a diet of fish oil containing Pufanox® were somewhat higher (though not significantly) than after fish oil alone. The ratio between 6-keto-$PGF_{1\alpha}$ and $TxB_2$ tended to be higher after fish oil with Pufanox® than after fish oil without Pufanox® or in controls.
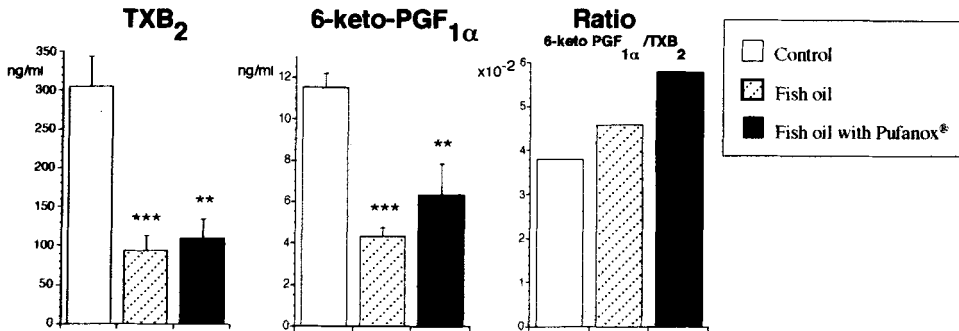


**Fig 2.** Effects of 18 days of dietary supplementation with fish oil with or without Pufanox® on in vivo production of $TxB_\alpha$ and 6-keto-$PGF_{1\alpha}$ and the 6-keto-$PGF_{1\alpha}$/$TxB_2$ ratio in the serum. Mean ± SEM, $n=6$ in each group. *$p<0.05$, ***$p<0.01$ and ***$p<0.001$ compared with controls.

**Triglycerides and cholesterol in plasma.** The triglyceride and cholesterol concentrations in plasma were markedly decreased in rats fed fish oil (with and without Pufanox®) compared with controls. No differences in triglyceride or cholesterol concentrations could be discerned between the two types of fish oil diet (Fig. 3).
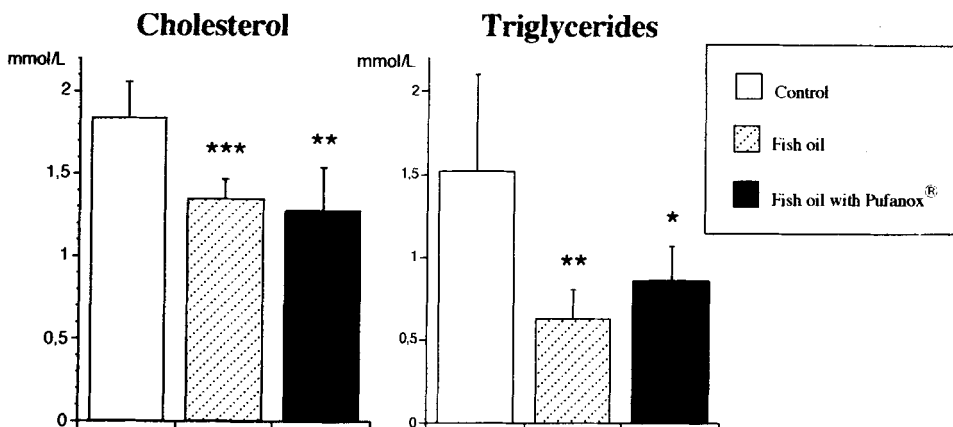


**Fig 3.** Effects of 18 days of dietary supplementation with fish oil with or without Pufanox® on cholesterol and triglycerides (mmol/L) in plasma. Mean ±SEM, $n=6$ in each group. *$p<0.05$, **$p<0.01$ and *** $p<0.001$ compared with controls.

**Vitamin E in myocardium and plasma.** After 18 days of dietary supplementation with fish oil, the vitamin E concentrations in myocardium were significantly lower in rats given fish oil without Pufanox® ($p<0.001$) and with Pufanox® ($p<0.05$), than in the control group. Vitamin E levels were somewhat higher in rats fed fish oil with vis-à-vis without Pufanox®, but the difference was not significant (Fig. 4). In plasma, the vitamin E levels were lower in rats fed fish oil with and without Pufanox® than in the control group.
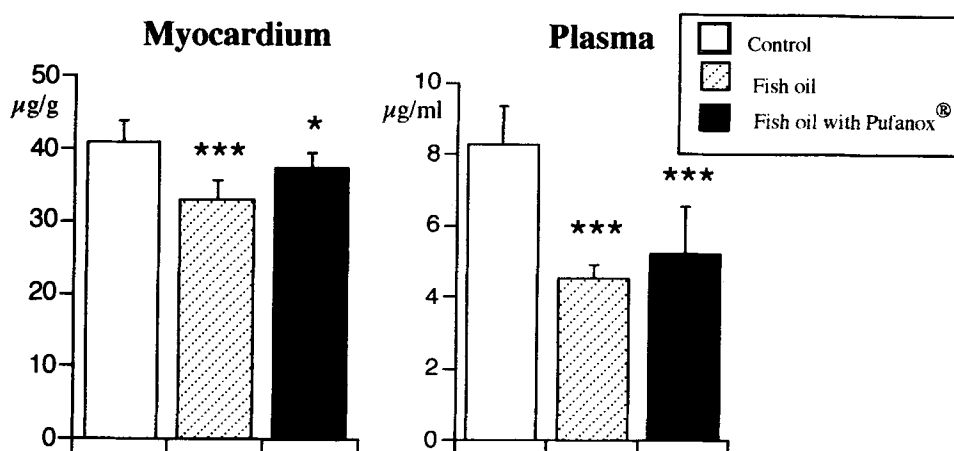


Fig 4. Effects of 18 days of dietary supplementation with fish oil with or without Pufanox® on vitamin E in rat heart (μg/g) and in plasma (μg/ml). Mean ±SEM, $n=6$ in each group. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with controls.

## Experiment II

**Vitamin E in myocardium and in plasma.** After 14 days of intake of water supplemented with vitamin E, the myocardial vitamin E levels were significantly higher in rats given 30 IU vitamin E daily than in the control group. The vitamin E levels in myocardium were somewhat higher in rats given 10 IU vitamin E daily than in rats given only pure tap water, but the difference was not significant. Although the vitamin E levels in plasma were somewhat higher in rats given 10 IU or 30 IU vitamin E than in the control group, the difference was not significant in either group (Fig. 5).
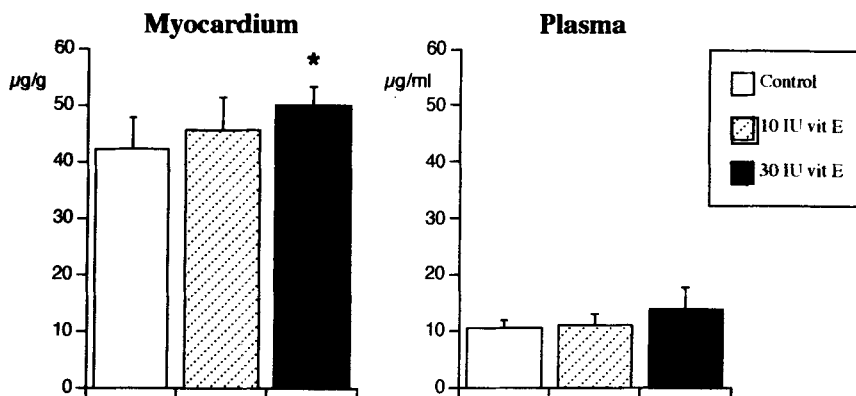
## Myocardium    Plasma



**Fig 5.** Effects of 14 days intake of water supplemented with 10 IU or 30 IU vitamin E daily on vitamin E in the rat heart (μg/g) and in plasma (μg/ml). Mean ± SEM, $n$=4 in each group. *$p$<0.05, **$p$<0.01 and ***$p$<0.001 compared with controls.

**Metabolite concentrations in heart and serum/plasma.** There were no significant differences in TxB$_2$, 6-keto-PGF$_{1\alpha}$, myocardial MDA, triglycerides or in cholesterol levels in myocardium or serum/plasma, compared with control rats (Table I)

**Table I.** Heart and serum metabolite concentrations in rats given drinking water supplemented with 10 IU or 30 IU vitamin E daily for 14 days[1]. Control rats received only normal tap water.

|  | Control | 10 IU vit.E | 30 IU vit.E |
| --- | --- | --- | --- |
| Myocardial MDA (nmol/g) | 35.7±4.2 | 37.6±5.8 | 39.0±6.1 |
| TxB$_2$, serum (mg/L) | 136.2±46.8 | 145.9±78.9 | 140.9±76.5 |
| TxB$_2$, myocardium (ng/mg) | 14.9±6.2 | 14.1±4.3 | 21.2±14.6 |
| 6-keto-PGF$_{1\alpha}$, serum (mg/L) | 9.1±2.3 | 8.8±3.2 | 8.9±3.5 |
| 6-keto-PGF$_{1\alpha}$, myocardium (ng/mg) | 92.9±27.4 | 113.1±133.8 | 204.2±170.3 |
| Cholesterol (mmol/L) | 2.1±0.1 | 1.9±0.4 | 2.0±0.3 |
| Triglycerides (mmol/L) | 1.9±0.5 | 1.4±0.3 | 1.5±0.2 |

[1] Values are means ± SEM (n=4 in all groups).

# DISCUSSION

Addition of Pufanox® to fish oil, as used in the present investigation, has been shown to markedly increase its stability after exposure to air in vitro (8). In the present investigation we found that addition of Pufanox® to the fish oil also reduced lipid peroxidation in vivo, measured as increase in malondialdehyde in plasma and myocardium. As lipid peroxidation in the blood or in the organs is regarded as a serious adverse effect of fish oil treatment, these findings may be very important for treatment with fish oil in the future.

Treatment with fish oil also tended to increase the prostacyclin/thromboxane ratio, measured as the ratio between the stable metabolites 6-keto-$PGF_{1\alpha}$ and thromboxane $B_2$, especially after fish oil supplemented with Pufanox®. Thromboxane $A_2$ is a major cyclo-oxygenase product synthesized from arachidonic acid especially in platelets and has potent vasoconstrictive and platelet-aggregating effects. Prostacyclin ($PGI_2$), in contrast, has vasodilatory and antiaggregatory effects and a high prostacyclin/thromboxane ratio is regarded as beneficial, especially in coronary artery disease. Eicosapentaenoic acid (EPA, 20:5, $n$-3) inhibits the synthesis of arachidonic acid, the precursor of thromboxane and prostacyclin, and competes with arachidonic acid in membrane phospholipids which are the major causes for the decrease in both thromboxane and prostacyclin production seen in the present investigation. EPA also gives rise to two metabolites, thromboxane $A_3$ and $PGI_3$, which were not measured in the present study. As thromboxane $A_3$ is inactive and $PGI_3$ has the same effect as $PGI_2$, the increase in these metabolites should further improve the prostacyclin/thromboxane ratio after fish oil.

The decrease in vitamin E content especially in plasma, but also in heart, shows that the vitamin E content in the fish oil used, 1.7 IU/g, was not high enough in this model. This is in agreement with results from human studies showing a better effect of large doses (30 ml daily) of a stable fish oil supplemented with 4.5 IU/g vitamin E, compared with a stable fish oil containing 1.5 IU/g of vitamin E. Thus only the fish oil having a larger content of vitamin E counteracted the blood glucose increase that is usually seen after fish oil ingested, and which may be due to increased lipid peroxidation in the pancreatic beta cells, thereby impairing insulin production or release (5).

The intake of fish oil strongly depressed both cholesterol and triglyceride levels in plasma in the present study. The mechanism underlying the triglyceride-depressive effect of omega-3 fatty acids is mainly a decrease in the hepatic synthesis of triglycerides, LDL and VLDL particles (2, 6).

In humans, the vitamin E content of the fish oil influenced the decrease in triglycerides (1), while the stability of the fish oil influenced the decrease in LDL-cholesterol; the higher the vitamin E content or stability, the greater the decrease in triglycerides and cholesterol. The reason why no

difference between the two fish oils was observed in the present study was probably that an insufficient amount of vitamin E was used.

Vitamin E given alone in two different doses had no effect on thromboxane, prostacyclin or blood lipids, indicating that the major effect of fish oil is due the oil itself rather than to the added vitamin E.

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