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***In vitro* and *in vivo* bimodal effects of docosahexaenoic acid supplements on redox status and platelet function**

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Abstract

Docosahexaenoic acid (DHA) is a prominent nutrient of marine lipids. Together with eicosapentaenoic acid, it is recognized as a protective molecule against atherosclerosis and thrombosis through the regulation of blood cell functions, especially platelets. Its high unsaturation index may however make it prone to peroxidation, which is usually considered as deleterious. This short review takes into consideration this possibility related to DHA concentrations both *in vitro* and *in vivo*. It is suggested that protective effects of DHA on platelet activation depend on the reduction of oxidative stress, and appear bimodal with the abolishment of such a protection when DHA is used at relatively high concentrations.

Introduction

Docosahexaenoic acid (DHA) is one of the long-chain polyunsaturated fatty acids (PUFA) of the omega-3 family found in marine lipids. Together with eicosapentaenoic acid (EPA), DHA is well-known for its anti-atherothrombotic potential, in particular by decreasing the arachidonic acid (ArA) content of blood cells [1], inhibiting thromboxane production in platelets, as well as leukotriene B₄ formation in leukocytes [2].

In addition to these inhibitory effects on ArA metabolism, DHA may decrease athero-thrombogenesis through the action of its lipoxygenase (LOX) end-products. Mono-hydroxylated derivatives produced through 12-, and 15-LOX, namely 11-, 14-, and 17-OH-22:6 (11-, 14-, and 17-HDoHE), respectively, inhibit the thromboxane-induced platelet aggregation (induced by the stable thromboxane A₂ receptor agonist U-46619), even stronger than 12-OH-20:4 and -20:5 (12-HETE and -HEPE) [3]. More recently, the double lipoxygenase product of DHA, 10(S),17(S)-diHDoHE, named protectin DX, has been shown to inhibit platelet aggregation through the inhibition of cyclooxygenase-1, which initiates prostanoid synthesis including the potent pro-aggregatory thromboxane A₂, as well as the response to the thromboxane A₂ mimetic U-46619 [4]. In addition, DHA is known to be oxygenated into several di/tri-OH derivatives (protectin D1, maresins, and resolvins) that are potent inhibitors of the inflammation process [5].

Supplementation of humans with long-chain omega-3 PUFA is usually considered as a positive approach to decrease the risk of atherosclerosis and thrombosis [6], as recently reviewed [7], although some controversies arose in the recent years [8]. Among the controversial issues are the intake doses of those highly unsaturated PUFA, which may be prone to peroxidation [9]. We have been especially interested in the effects of different concentrations of DHA upon blood platelets and their blood environment both *in vitro* and *in vivo*, in particular regarding the redox status.

***In vitro* effects of DHA on platelet reactivity and redox status**

When compared with EPA, DHA pre-incorporated in human platelet phospholipids was more potent to inhibit platelet aggregation induced by various agonists. While EPA was substantially released, and oxygenated like endogenous ArA in response to thrombin, DHA was preferentially transferred from phosphatidylcholine to phosphatidylethanolamine [10], especially their plasmalogen subclass [11]. One possible mechanism for the high potential of DHA to inhibit thrombin-induced platelet aggregation is the strongest inhibition of endogenous ArA oxygenation by cyclooxygenase, while the lipoxygenase activity was not altered [12]. The cyclooxygenase inhibition by DHA was however alleviated by doubling the concentration of DHA added to platelets, without altering the inhibition of

platelet aggregation, presumably because prostaglandin D₂ formation, a potent inhibitor of platelet aggregation, was increased [13]. We might speculate that such a paradoxical effect might be explained by the oxidative stress induced by high concentrations of DHA, which could have increased the cyclooxygenase activity (see later results below). In addition to the inhibition of platelet cyclooxygenase by DHA enrichment, the response of DHA-rich platelets to the thromboxane A₂ mimetic U-46619 was strongly inhibited through the decreased affinity of this agonist to its membrane receptor sites [14]. Another platelet function, the pro-coagulant activity of platelet phosphatidylserine exposure in response to collagen, can be altered by DHA (or EPA) enrichment *in vitro*. Again, the inhibitory effect of those PUFA, only significant at 0.75 mM, was not increased by doubling their concentration [15].

Considering the high unsaturation of long-chain omega-3 PUFA, especially DHA, it is likely that lipid peroxidation will be favored when their concentrations are increased in biological systems. Indeed, PUFA, and in particular DHA, increase free radical formation in oxidative stress conditions [9]. We have been interested in the dose-related effect of DHA on human platelet redox status after *in vitro* enrichment. A dose-dependent increased DHA concentration in membrane phospholipids (phosphatidylcholine and phosphatidylethanolamine) after incubation with albumin-bound DHA (DHA/albumin ratios of 0.01, 0.1, and 1, corresponding to 0.5, 5, and 50 μM) was observed but, at the lowest concentration, this was only significant in the plasmalogen ethanolamine subclass. This observation has been discussed regarding the potential antioxidant activity of the plasmalogen subclass [16]. Malondialdehyde (MDA) and the 12-lipoxygenase end-product 12-HETE, measured as markers of oxidative stress, were significantly increased after the highest enrichment, but significantly decreased at the lowest one. These results suggest a bimodal effect according to DHA concentrations, with an interesting protection against lipid peroxidation at low concentrations [16]. The increased peroxidation observed after the highest enrichment was in agreement with significant decreased platelet alpha- and gamma-tocopherols.

***In vivo* effects of DHA on platelet function and their blood environment**

As a component of long-chain omega-3 PUFA, DHA intake has long been reported a protective agent against atherosclerosis and thrombosis as recently reviewed [6, 7, 17, 18], acting through several ways. One of them is the inhibition of blood platelet activation *via* mechanisms already mentioned above such as competition with ArA metabolism, especially thromboxane formation. However, meta-analyses revealed heterogeneities in results that might be due to different intake doses [8], and high daily supplementation may decrease antioxidant defenses such as alpha-tocopherol in plasma [19]. Considering the deleterious effect of oxidative stress in platelet [20-23] and blood mononuclear cell functions [24], we have investigated the relationship between the doses of DHA intake and the reactivity of those cells.

In a study with healthy human volunteers, increasing doses (200, 400, 800, and 1600 mg/day) of DHA in triacylglycerols from micro-algae were consumed for two-weeks each dose. There was a dose-dependent increase of DHA in plasma phospholipids, as well as in platelet phosphatidylcholine and phosphatidylethanolamine. Whether, the increase in phosphatidylethanolamine reflected a preferential incorporation within their plasmalogen subclass, as found *in vitro* (see above), was not investigated. In terms of platelet reactivity, the dose-response was bimodal with a maximal inhibition of platelet aggregation and cyclo-oxygenation of exogenous ArA after 800 mg/day DHA. After 1600 mg/day DHA, the aggregation was less inhibited and cyclo-oxygenase activity was not significantly altered [25]. In addition, platelet vitamin E (alpha-tocopherol) significantly increased after 200 mg/day DHA. In agreement with this increased antioxidant status in platelets, urinary isoprostanes (8-epi-PGF_{2α}), as a marker of the global redox status, were significantly decreased after this low dose intake of DHA, whereas they were significantly increased after 1600 mg/day DHA [25]. This clearly suggests that low dosages of DHA are protective against platelet-related atherothrombotic risk in relation with an antioxidant effect at low dosage of DHA, whereas the highest dose tested (1600 mg/day DHA) was less potent against platelet reactivity, possibly because of an increased oxidative stress.

Plasma low-density lipoproteins (LDL) were also studied in response to the different doses of DHA. LDL cholesterol esters and phospholipids were dose-dependently enriched in DHA according to the intake doses. However, a bimodal effect of this DHA enrichment could be observed, with a significant increase of LDL alpha-tocopherol content, and decrease of LDL MDA after 200, 400, and 800 mg DHA/day, but not after 1600 mg. Interestingly, the lag phase in the copper-induced oxidation of LDL was similarly increased after those three doses but not after the highest dose, meaning that the intermediary doses of DHA may protect against LDL oxidation. Although not significantly different from the other doses, the most active dose in such a protection, and MDA decrease was observed after 400 mg/day DHA [26].

In parallel, the apoptosis of blood mononuclear cells induced by oxidized LDL was tested. While there was a dose-dependent correlation in the IL-2 transcript expression with DHA intake, monocyte apoptosis was significantly attenuated after 400 mg/day DHA, and such a protective effect was maintained at higher doses, although to a lesser extent [27].

Altogether, these results obtained in healthy volunteers strongly suggest that daily doses of DHA lower than one gram should be recommended to decrease the athero-thrombotic risk related to blood cell reactivity, especially platelets. Several markers even suggest that the most active daily doses of DHA might be lower than 500 mg. In agreement with these dose-effect investigations in healthy volunteers, it has previously been shown that low daily doses of DHA + EPA (150 + 30 mg) exhibit the same protective effects in elderly people, through a reduction of oxidative stress [28]. The effects of different intake doses of DHA in healthy human volunteers has been recently reviewed [29]. Interestingly, and more recently, the intake of 400 mg/day DHA in type-2 diabetic patients has shown similar protection, with even more potency than in healthy volunteers. Indeed, platelet aggregation was decreased by 50%, several markers of oxidative stress were significantly decreased, and vitamin E was significantly increased in both blood plasma and platelets [30]. This suggests that the higher the oxidative stress, the higher the efficiency of DHA moderate intake to decrease such a stress.

The mechanism underlying such a protection through a decreased oxidative stress is not clear. We might however speculate that DHA being easily oxidized, it might have a trapping effect of oxidant species at low doses, then saving vitamin E as observed in several studies, although participating in the oxidative stress at high concentrations. In favor of this hypothesis is the observation that a dose-dependent formation of 4-hydroxy-hexenal, one specific peroxidation by-product from omega-3 PUFA, was observed after DHA intake, while 4-hydroxy-nonenal, the counter peroxidation by-product from omega-6 PUFA, did not change [26].

Conclusion

The long-chain omega-3 PUFA DHA and EPA, in particular DHA, are useful nutrients to protect against the athero-thrombotic risk associated with blood platelet reactivity. DHA effect at moderate concentrations, both *in vitro* and *in vivo*, might be related to reduction of oxidative stress (Figure 1). This would be especially useful in situations where the redox status is altered toward oxidation such as aging and diabetes.

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Figure 1. Summary of results observed after DHA intake in humans and dose-related effects of DHA on human blood platelets.

For clarity, this figure only relates with low and high doses of DHA (see text for details), and shows the variations in malondialdehyde (MDA), as a global marker of oxidative stress, and vitamin E, as the main antioxidant lipid soluble vitamin.

In all DHA enrichment approaches, DHA was significantly increased in main platelet glycerophospholipids (PC and PE). This enrichment was higher after high doses compared to low doses.

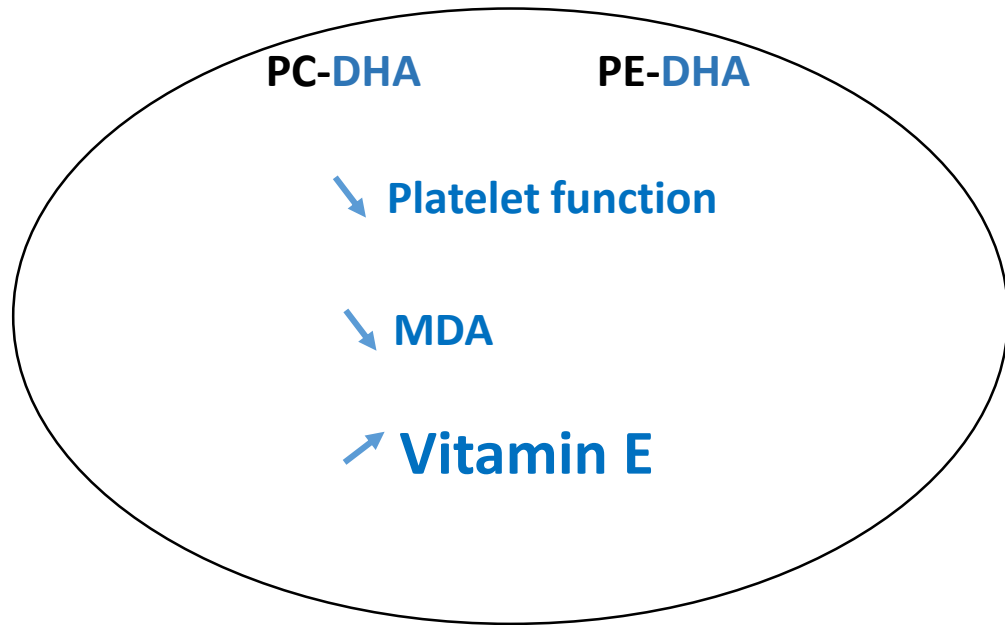
Highlights

Docosahexaenoic acid exerts bimodal effects on blood platelet aggregation, oxidized-induced mononuclear cells apoptosis.

Docosahexaenoic acid at low concentrations increase vitamin E whereas high concentrations tend induce oxidative stress.

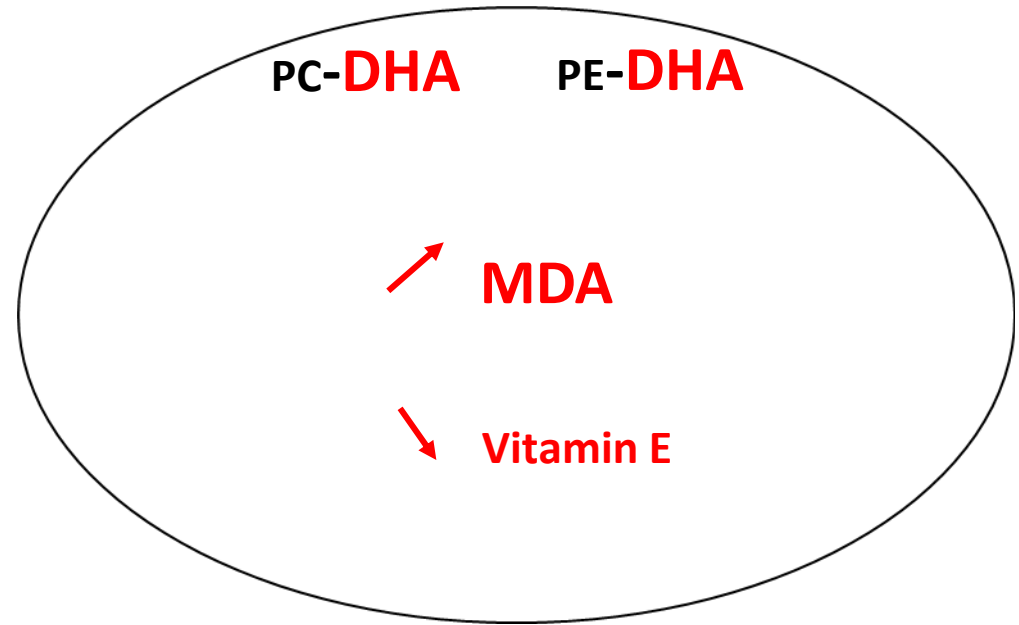
Low doses of docosahexaenoic acid down regulate platelet activation and oxidative stress in diabetic and elderly subjects.

Low dose of DHA



Both *in vitro* and *ex vivo* studies (elderly and healthy adults, diabetic patients)

High dose of DHA



Both *in vitro* and *ex vivo* studies (Healthy adults)